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SHAPE TRANSFORMATIONS IN THE RED CELLS OF CAMELS, BIRDS, REPTILES, AMPHIBIA, AND FISHES

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The bearing of shape changes on the problem of red-cell structure is so great that we cannot afford to omit studying the transformations which occur in oval nucleated red cells in order to compare them with those in the discoidal non-nucleated red cells of man and of most mammals. The purpose of this paper, accordingly, is to give a description of the shape changes observed in the erythrocytes of camels, birds, reptiles, amphibians, and fishes.

In order to express the results in a small compass, I have condensed them into two tables and a series of observations which deal with points which require explanation or which ought to be put on record for the benefit of other investigators in the field.

OBSERVATIONS ON THE TABLES

(1) *Material.* As examples of the various vertebrate orders, I have used the camel (*Camelus bactriens*), the pigeon (*Columba livia*), the turtle (*Pseudemis elegans*), the frog (*Rana esculenta*), and the carp (*Carassius auratus*). The blood, drawn from a vein or from the heart, was received into heparin, and the measurements and other manipulations of the cells were made without delay.

(2) *Measurements.* The measurements of cell length and breadth, and of nuclear length and breadth, were made by the photographic method of Ponder & Millar (1924), with critical illumination and at a magnification of 480. No attempt was made to measure the thickness of the cells as seen on edge, for this is exceedingly difficult for technical reasons. Instead, the volume of the cells was found by dividing the percentage volume (high-speed hematocrit) by the red-cell count, and from the value for the volume, together with the figures for cell length $2a$ and the cell breadth $2b$, the mean thickness can be calculated. The cell area is approximately $2\pi ab$. All measurements are given in μ.

A real difficulty arises in connexion with these measurements, for the red cells in birds, reptiles, amphibians, and fishes probably do not constitute an entirely homogeneous population. In birds, Kennedy & Climenko (1928) describe two types of cell, one with an oval nucleus and the other with a round nucleus, and I think that one can go further than this, and distinguish three types in fresh preparations: (i) oval cells with nuclei of varying degrees of eccentricity (the extent of which may be greatly exaggerated in fixed and stained films), (ii) oval cells with round nuclei, and (iii) round cells with round nuclei. The round cells with round nuclei are probably the younger cells, for they have an open nuclear structure and usually show diffuse basophilia; this is the view taken by Bizzozero (1890) and by Kennedy & Climenko. In camels, Loo (1929) described a large round type of red cell in addition to the prevailing oval type, Cullen (1903) describes both oval and round cells in the blood of the skate and the dog fish, and in an early

monograph (Ponder, 1924) I have referred to four types of erythrocyte in birds and to three types in reptiles, amphibians and fishes. It happens that the adult oval cell predominates (more than 90% of the cells seen), and so the measurements in Table 1 refer to this type. An investigation of the distribution of red-cell dimensions and an elaborate statistical treatment will be required before a complete description of red-cell shape in the lower vertebrates is available. Meantime we may remark upon the fact that young red cells (reticulocytes and erythrocyte precursors in the bone marrow) tend to be round, the distinctive shape characteristic of the animal (the biconcave disk in most mammals, the ovalocyte in some individuals, the oval cell in the camels, and the oval nucleated cell in the lower vertebrates) being fully exhibited in the adult red cell only.

(3) *Slide and slip shape transformation.* This shape transformation, due to the diffusion of alkali from glass surfaces and the transference of an 'anti-sphering' albumin from the cell surface to the glass by adsorption (Furchgott & Ponder, 1940), is peculiar to the discoidal mammalian red cell and the ovalocyte. Other types of cell show no shape change except that corresponding to slight crenation; this consists of a mottling of the surface, not unlike that described by Millar (1925) in the mammalian ghost, together with wrinkles running out radially from the nucleus and terminating in fissures at the cell margins. A fine saw-toothed scalloping of the margins also occurs, but none of these changes are in any way comparable to the almost complete loss of shape observed in the crenated mammalian erythrocyte. Cells in saline seem to stick to the slide more readily than do cells in plasma.

(4) *Effect of lecithin.* The addition of an equal volume of a lecithin sol to a suspension of mammalian red cells in saline or in plasma causes a rapid transformation of disks to perfect spheres, irrespective of whether the cells are disks or ovalocytes (Ponder, 1939). No corresponding change occurs in the cells of camels or of pigeons, turtles, frogs, or fish, although a shape change which I call 'lanceolation' can sometimes be observed in the nucleated erythrocytes as a result of the addition of lecithin. When this change occurs, the cells lose their oval outline and become shaped like grains of wheat, with sharp ends and an almost diamond-shaped cross-section; sometimes this change occurs symmetrically, and sometimes one end of the cell remains oval while the other develops a lanceolate point. The extent of this lanceolation probably depends on the concentration of lecithin effective at the cell surface, for it is certainly quite variable throughout the preparation when a lecithin sol is run in under the cover-slip in the usual way and it is often entirely absent. If large amounts of lecithin are added, some of the cells may become prolytic forms, and ultimately haemolyse.

In the case of avian red cells at least, this lanceolation is increased by running pigeon plasma under the cover-slip, and for this reason I think that it bears little relation to the disk-sphere transformation of mammalian erythrocytes, which is reversed by plasma.

(5) *Effects of rose bengal.* Washed mammalian disks and ovalocytes are promptly converted into spheres by rose bengal in concentrations 10^{-6} M when illuminated, and the same shape change occurs rapidly in higher concentrations of the dye in the dark. The oval red cells of the camels and of the birds, reptiles, amphibians and fishes show no such immediate change, although they may show lanceolation. After some minutes (depending on the dye concentration, the intensity of illumination, etc.) the red cells of the mammals (including the ovalocytes and the cells of the Camelidae), and the cells of the pigeon also, become first less oval and then spherical, and show the characteristic

homogeneous appearance and bright peripheral diffraction rings of the prolytic sphere. These shape changes occur in the cells one by one, without any apparent change in volume, and the process can be hastened by opening the iris diaphragm and flooding the cells with light. Each prolytic sphere ultimately haemolyses, losing its pigment and at the same time becoming an oval ghost.

The red cells of the turtle, the frog, and the carp, on the other hand, may first become lanceolate, but then turn into circular and flat prolytic disks or plates. There is great variation in this prolytic shape change; some cells lose their pigment with scarcely any alteration in shape, others form oval disks, still others circular disks, and a few form what are apparently perfect prolytic spheres. The diversity is so great that one suspects that one is dealing with a mixed population in which there are differences of kind as well as of degree. These prolytic forms are flat even up to the moment of losing their pigment, and in this respect they differ from the prolytic form in birds, where the element of flatness is lost before lysis. As an extreme instance, some of the red cells of the frog are haemolysed by rose bengal without any shape change at all.

(6) *Saponin and bile salts.* After a few minutes the discoidal cells of mammals and the mammalian ovalocyte become prolytic spheres following the addition of saponin or bile salts. The red cell of the camel diminishes in its long axis, acquires a dull homogeneous appearance, and shows brilliant diffraction bands at its edges; this prolytic spheroid finally haemolyses, sometimes without a further change in shape, and sometimes after becoming a prolytic sphere. The pigeon red cell shows similar change, becoming either a sphere or an oval spheroidal body. The red cell of the turtle, the frog, and the carp, on the other hand, do not lose their element of flatness; as looked down upon, they remain ovals or become round, but even at the moment of haemolysis they are flat plates, with their *c*-axis apparently unchanged. Some of these shape changes have been described by Shattuck (1928), who has pointed out that sodium oleate breaks down both the cell membrane and the nuclear membrane, whereas saponin and sodium taurocholate break down the cell membrane only.

(7) *Hypotonic plasma.* All types of red cell swell in hypotonic plasma, behaving as more or less perfect osmometers. Oval cells become first lemon-shaped and later appear lemon-shaped or spherical. They finally haemolyse, leaving oval, flat, ghosts with a highly refractile nucleus and a very indistinct outline. The oval form of the ghost is assumed at the moment of haemolysis.

(8) *Form of the ghost.* Irrespective of the shape changes which precede lysis, the form of the haemolysed ghost is always substantially that of the red cell from which it is formed. This form is assumed at the moment of haemolysis, and constitutes very strong evidence for the existence of a pre-formed structure either at the surface or in the interior (see Ponder, 1942). The dimensions of the ghost are always smaller than those of the original cell. In the case of the pigeon, for example, the cell length is $15\,\mu$ and the cell breadth is $8{\cdot}6\,\mu$; the ghost is $9{\cdot}5\,\mu$ long and $6{\cdot}3\,\mu$ broad. So far as can be gathered from photographic measurements which are not altogether satisfactory, the size of the nucleus in unchanged.

Table 1

<table>
<tr><th></th><th>Man</th><th>Ovalocyte</th><th>Camel</th><th>Pigeon</th><th>Turtle</th><th>Frog</th><th>Carp</th></tr>
<tr><td rowspan="2">Shape</td><td colspan="2">Biconcave</td><td rowspan="2">Biconvex Oval</td><td colspan="4" rowspan="2">Biconvex nucleated Oval</td></tr>
<tr><td>Disk</td><td>Oval</td></tr>
<tr><td>Slide and slip in saline</td><td colspan="2">Crenation and spheres</td><td colspan="5">None</td></tr>
<tr><td>Crenation in saline</td><td colspan="2">Variable, coarse and fine</td><td>Surface mottling</td><td colspan="4">Radial wrinkling, surface mottling, saw-tooth edges</td></tr>
<tr><td>Lecithin sol</td><td colspan="2">Spheres</td><td>None</td><td colspan="4">Usually none; occasional lanceolation</td></tr>
<tr><td>Rose bengal immediate</td><td colspan="2">Spheres</td><td>None</td><td colspan="4">Lanceolation</td></tr>
<tr><td>Rose bengal later</td><td colspan="4">Prolytic spheres</td><td colspan="3">Prolytic round or oval plates</td></tr>
<tr><td>Saponin and bile salts</td><td colspan="2">Prolytic spheres</td><td colspan="2">Prolytic spheres and ovals</td><td colspan="3">Prolytic round or oval plates</td></tr>
<tr><td>Hypotonic plasma</td><td colspan="2">Prolytic spheres</td><td colspan="5">Prolytic ovals and spheres</td></tr>
<tr><td>Form of ghosts</td><td>Flat disk</td><td>Oval plate</td><td colspan="5">Oval plates</td></tr>
</table>

Table 2

	Man	Ovalocyte	Camel	Pigeon	Reptile	Frog	Carp
Cell length	8·6	9·1	8·0	15·0	22·9	28·0	14·5
Cell breadth	—	5·5	4·4	8·6	15·0	16·5	10·5
Nuclear length	—	—	—	5·8	8·3	8·9	6·3
Nuclear breadth	—	—	—	4·1	4·5	6·2	3·9
Area	160	110	55	200	540	750	240
Volume	86	73	29	117	365	900	175

CONCLUSIONS

1. The almost instantaneous, reversible, disk-sphere transformations (slide and cover-slip, lecithin, and rose bengal) occur only in the biconcave red cells of mammals (including ovalocytes). They do not occur in the biconvex red cells of the Camelidae, nor in any type of nucleated erythrocyte.

2. All types of red cell show prolytic changes (saponin, bile salts, high concentrations of rose bengal, hypotonicity). As we ascend from the fish to the mammal, the prolytic form shows an increasing departure from the original shape and an increasing approach to the form of a perfect sphere. Thus in fishes the prolytic form is an oval, or sometimes round, plate (at least two axes unequal), while in mammals it is a sphere (all three axes equal).

3. The two foregoing conclusions mean that the structure responsible for the red-cell shape is more labile in the higher vertebrate forms, in which it seems to be an essentially surface structure, than in the lower vertebrate forms, in which it seems to be an organized cytoplasm in the usual sense of the term.

4. The form of the ghost is always essentially that of the red cell from which it is derived, and this form is assumed at the moment of haemolysis. The sequence of shape changes suggests that the inherent structure of the cell (and of the ghost) is subjected to the action of obscure forces which develop during the haemolytic process, and which disappear when lysis takes place.

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QUANTITATIVE ASPECTS OF THE DISK-SPHERE TRANSFORMATION PRODUCED BY LECITHIN

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(With One Text-figure)

This paper is concerned with quantitative aspects of the disk-sphere transformation of the mammalian red cell produced by lecithin (Ponder, 1935 *a*). This shape transformation, brought about when lecithin is added to mammalian red cells in saline or in plasma, and reversed by washing off the lecithin or by adding an excess of plasma, is very suitable for quantitative study, since, unlike the shape transformations produced by rose bengal and other lysins, it is not soon followed by haemolysis. Exact work on the factors involved, however, has been very difficult, for two reasons. (1) An unrelated disk-sphere transformation occurs when red cells in saline, whether lecithin-treated or not, are placed between a slide and a closely applied cover-glass (Ponder, 1929), and so it has not been possible to examine the lecithin shape transformation of the cells in saline except in uncovered preparations or in hanging drops. Even under these circumstances, the two factors responsible for the slide-slip transformation, removal of anti-sphering substance by the glass and diffusion of alkali from the glass (Furchgott, 1940; Furchgott & Ponder, 1940) interfere with the observations. This difficulty can now be avoided by using plastic slides and cover-slips, between which these interfering phenomena do not occur. (2) The original method of adding lecithin to plasma or saline by mechanical emulsification is not satisfactory, as the amount of lipoid emulsified is usually unknown and its physical state is variable. Recently I have used sols of lecithin in saline, in which the dispersion approaches uniformity and a known quantity of lipoid is present.

With plastic slides and cover-slips and with lecithin sols the regularity with which the shape changes occur are greatly increased and the phenomena become susceptible of detailed quantitative study.

1. PREPARATION OF SOLS AND DETERMINATION OF THEIR POTENCY

Lecithin* is dissolved in ethyl alcohol in the proportion of 0·1 g. to 10 ml. In a large flask 100 ml. of 1 % NaCl is brought to the boil, and to it is added, drop by drop and with swirling, 1·0 ml. of the alcoholic solution of lecithin. The sol is boiled for a few minutes to drive off the alcohol, cooled slowly, and kept in the refrigerator. Sols containing up to four times this quantity of lecithin can be prepared, and are fairly stable; less concentrated sols are made by dilution with 1 % NaCl.

* The lecithin used in these experiments was 'lecithin ab ovo', Merck, but identical results have been obtained with almost pure white lecithin prepared from eggs in the laboratory. Distearyl-lecithin, a sample of which was kindly given to me by Dr Harry Sobotka, is virtually insoluble in saline, and does not produce shape transformations.

A series of dilutions of the sol in 1% NaCl, 1 in 2, 1 in 4, ..., 1 in 64, is prepared, and 1 ml. of each is added to a series of small vials, the first containing 1 ml. of undiluted sol. Other vials, containing 1 ml. of sols with twice, three times, or four times the amount of lecithin as in the standard sol ($2x$ sol, $3x$ sol, $4x$ sol, etc.) can be added to the series as needed. To each vial is added 0·25 ml. of blood or of washed red-cell suspension, the volume concentration or red-cell count being known. After mixing and standing at room temperature (22° C.) for 15 min., a drop is taken from each vial, placed on a plastic slide, covered with a plastic cover-slip,* and examined with a high dry objective and a 10 × eyepiece. Observations should also be made at longer and at shorter times, but after 15 min. the effects produced by the lecithin are substantially complete.

The preparations from the various vials show predominantly one of four forms: (i) perfect spheres, (ii) crenated spheres, (iii) crenated disks, and (iv) more or less perfect disks. Attempts at a more elaborate classification result in confusion, for any one preparation may contain a few crenated spheres along with a large majority of perfect spheres, a few crenated disks when most of the cells have a clearly recognizable biconcave form, and so on. In general, what we require is the smallest quantity of sol which produces a given shape change (crenated disk, perfect sphere, etc.) in the preparation under the particular conditions of the experiment.

2. THE QUANTITY OF LECITHIN REQUIRED FOR SPHERING

(i) *Minimal values for human red cells.* Since the concentration of lecithin in these sols is too small to be conveniently determined by chemical methods, the amount needed to produce sphering is best determined by preparing a number of sols of different concentrations and finding which of them produce perfect sphering and which do not. One result can be given as typical of a number of determinations. Using 1 ml. of a suspension of normal, thrice washed human red cells in saline, with $4{\cdot}6 \times 10^9$ cells per ml., I find that I need to add 5 ml. of a sol containing 50γ/ml. in order to get perfect spheres within 15 min.; a sol containing half this amount does not produce perfect spheres even after 2 hr., the cells remaining at the crenated disk stage, while a sol of twice the concentration produces perfect spheres almost at once.

This value, 250γ of lecithin per $4{\cdot}6 \times 10^9$ red cells, is a common value found for human erythrocytes, and the method is sufficiently accurate to allow one to detect variations by a factor of as little as 2 in either direction. Determinations for the cells of 27 individuals show the mean quantity to be about 250γ of lecithin per 5×10^9 cells, the lowest value being 150γ, and the highest 400γ.

A lecithin sol containing 50γ/ml. corresponds to $0{\cdot}6 \times 10^{-4}$ M (taking the molecular weight of lecithin as 777), and, assuming molecular dispersion, this contains $3{\cdot}6 \times 10^{16}$ molecules. The quantity required for sphering 5×10^9 cells, 250, accordingly contains $1{\cdot}8 \times 10^{17}$ molecules. The surface of each cell being about $150\mu^2$, the entire surface presented by the suspension is about $7 \times 10^{11}\mu^2$, and so there are available about $2{\cdot}6 \times 10^5$ molecules per μ^2. If all the molecules were at the surface, each would require to cover about 28 A.^{2}, which is a very likely figure for the area which a lecithin molecule would cover when oriented with its fatty acid chains normal to the surface (distance between chains, about 5 A.). The amount of lecithin required to produce sphering is therefore

* Plastic slides and cover-glasses are made from sheets of inexpensive material (Turtox Plastic) obtainable from the General Biological Supply House, Chicago.

about the same as that required for a monolayer of oriented lipoid at the red-cell surface.

(ii) *The effect of temperature.* The smallest quantity of lipoid necessary to produce perfect sphering in 15 min. at different temperatures can be found by repeating the foregoing determinations at 5, 22, and 38° C., the suspension, the sol, the slides, and the stage of the microscope being cooled or warmed as the case may be. Experiments of this kind show clearly that there is a small, positive, temperature dependence, smaller quantities of lecithin being required at 38 than at 5° C. The difference involves a factor of 2 or less, e.g. $300\gamma/5 \times 10^9$ cells at 5° C. and $175\gamma/5 \times 10^9$ cells at 38° C., which gives a factor of 1·7. The temperature coefficient of the effect is therefore small and positive.

(iii) *The effect of fixation.* One of the most interesting aspects of the lecithin disk-sphere transformation is the fact that a certain definite quantity of lecithin is required to initiate it. There is accordingly something corresponding to a 'yield point' describable in terms of γ of lecithin. As the quantity of lipoid is increased from zero, the component of the membrane responsible for the discoidal shape (a postulated ultrastructure or fixed framework) is able to withstand whatever forces are associated with the concentration of lecithin until that concentration reaches a certain value; at this point the shape transformation begins to be completed if the concentration is further increased. As the value of the yield point should depend on the properties, e.g. the rigidity, of the ultra-structure, we may try to alter these experimentally with a view to finding what effect on the yield point results, and the simplest way of affecting the rigidity of the ultra-structure, either directly or by affecting the rigidity of the cell as a whole, is by the use of a fixing agent.

A series of concentrations of formol* in saline, from 0·2 to 1·0%, is prepared, and 1·0 ml. of these is placed in a series of vials. To each vial is added 0·2 ml. of washed red-cell suspension (5×10^9 cells/ml.). The cells are allowed to stand in contact with the formol for 30 min. at room temperature, and 1·0 ml. of a lecithin sol containing 100γ/ml. of lecithin is then added to each vial. The cells are examined after 15 min. and the results are expressed in the following way (*S*=sphere; *CS*=crenated sphere; *CD*=crenated disk; *D*=disk):

Table 1

Formol, %	0·0	0·2	0·3	0·4	0·5	0·6	0·8	1·0
Form of cell	*S*	*S*	*CD*	*D*	*D*	*D*	*D*	*D*

Suppose that in the absence of formol the largest quantity of lecithin which can be added to the cells without changing them from their discoidal form is 1·0 ml. of a lecithin sol containing 25γ/ml. (the usual figure, see above). After the action of 0·4% formol for 30 min., the discoidal form persists even though 1·0 ml. of a sol of four times this concentration, i.e. 100γ/ml., is added, although this quantity of sol spheres cells exposed to the action of 0·2% formol. The exposure to formol has accordingly raised the yield point. Repeating the experiment with a sol containing 200γ/ml. of lecithin, we obtain results like the following:

Table 2

Formol, %	0·0	0·2	0·3	0·4	0·5	0·6	0·8	1·0
Form of cell	*S*	*S*	*S*	*S*	*S*	*CD*	*D*	*D*

* Formol has been used as a representative fixative because I used it in an earlier investigation (Ponder, 1940). The results, however, are typical of those of fixatives in general. In this connexion, it is interesting to observe that the typical discoidal shape is maintained when red cells are suspended in 10% ethyl alcohol, and that disk-sphere transformations occur even in this medium.

After the action of 0·8% formol for 30 min., the discoidal form is maintained after 1·0 ml. of a sol with 200γ/ml. of lecithin has been added, although this quantity of sol spheres cells exposed to 0·5% formol and lesser concentrations. The yield point is thus greater when the cells are exposed to 0·8% formol than when they are exposed to 0·4% formol, as might be expected from the action of the fixative on the rigidity of the cell components.

(iv) *The effect of haemolysins.* I expected that exposure of the cells to sublytic concentrations of lysins such as saponin and the bile salts would decrease the yield point, just as exposure to fixatives increases it. No such effects have been observed, but the experiments are not very satisfactory because the lysins themselves bring about a reversal of the lecithin disk-sphere transformation, turning the spherical cells into perfect disks (see § 3, below). As a result, it is difficult to be sure of the end-points.

(v) *The effect of tonicity.* If washed human red cells are suspended in hypotonic NaCl of tonicity 0·75 or less, somewhat greater amounts of lecithin are needed to produce sphering than when the medium is isotonic. The difference is small, the factor involved being less than 2. Apart from this curious effect, alterations in tonicity are accompanied by the usual changes in the size of the lecithin spheres, those in hypotonic solutions being larger, and those in hypertonic solutions being smaller (Ponder & Robinson, 1934; Ponder, 1935*b*).

(vi) *Sphering of reticulocytes and poikilocytes.* If reticulocytes are stained in the usual way by adding 1 drop of blood to 1 ml. of 1% brilliant cresyl blue in saline, and if equal volumes of this preparation and of a lecithin sol are mixed, the process of sphering of the reticulocyte can be observed in wet mounts. These cells remain as deformed disks after the non-reticulated cells have become spherical, and it is apparent that the reticulum prevents sphering by its strands holding in the cell surface and producing puckers corresponding to the end of each strand. If a little saponin is run under the cover-slip so as to produce haemolysis, each reticulocyte fades, but the stained reticulum is left behind floating in the fluid with each strand in the same relative position as originally. There can be no question but that the reticulum is a 'solid' structure.

Poikilocytes are sphered by lecithin, but the intermediate forms are irregularly crenated, the major crenations corresponding in a rough way to the irregularities of the cell before sphering begins. A poikilocyte with a long tongue-shaped 'pseudopod' attached to a more or less discoidal body, for example, will show sphering of the body with retention of the pseudopod for a considerable time; the pseudopod, however, finally rounds off and is incorporated into the perfect sphere. As one observes the shape transformations of poikilocytes, one cannot help concluding that different parts of the cell offer different resistances to sphering, and that the regions of the pseudopods are the most resistant of all.

3. REVERSAL EFFECTS

The reversal of the lecithin disk-sphere transformation was described shortly after the description of the phenomenon itself (Ponder, 1933, 1935*a*), when it was found that the spherical forms become disks if the lecithin is washed off with isotonic NaCl or if plasma is added. Under such circumstances all the changes of the disk-sphere transformation occur in reverse order, and the transformation and its reversal can be repeated several times.

Washing and the addition of plasma, however, are not the only ways of producing reversal, as I found when studying the effect of sublytic quantities of lysins on the transformation and as is illustrated by the action of benzene in solution in isotonic NaCl on a red-cell preparation of spherical red cells made by the addition of a lecithin sol. As the benzene solution is run under the cover-slip, the cells rapidly assume the form of perfect biconcave disks; in this form they remain for some time until they become prolytic spheres under the haemolytic action of the benzene and finally haemolyse.

A similar reversal of the lecithin disk-sphere transformation, from sphere to disk, is produced by isotonic solutions in saline of chlorobenzene, brombenzene, dibrombenzene, indol, skatol, chloroform, ether, the bile salts, the straight-chain alcohols, and, in general, by solutions of substances which are lipoid solvents and which have been shown to have an affinity for the components of the red-cell membrane (Ponder, 1939; Ponder & Hyman, 1939; Ponder, 1941*b*). When the substance added is itself an active lysin (the bile salts and some of the alcohols) one is apt to miss the phase of reversal, because it is followed by the formation of prolytic spheres which may be mistaken for the original lecithin spheres.

Plasma and serum also bring about the reversal of the shape transformation, and in addition produce a very noticeable rouleau formation among the disks which are formed.

This reversal is so quantitative that the amount of a substance required to produce reversal and the amount of lecithin used to produce the spherical form can be titrated against each other with the red-cell shape as an indicator, much as one titrates an acid against an alkali. The reversing substance, e.g. 10 mM./l. benzene in isotonic saline, is placed in a burette, and the suspension of spherical red cells, made up of 2 ml. of lecithin sol and 0·2 ml. of a suspension of washed cells in saline, is placed in a test-tube. To insure uniformity of results, the lecithin and the cells should be allowed to stand together for 30 min. The cells in a drop of the suspension, placed between plastic slide and plastic cover-slip, are first examined to establish that they are perfect spheres. A small volume of the solution of the reversing agent (e.g. 0·2 ml.) is then added to the tube from the burette, and a drop of the system is examined again. If the cells are still spheres, a further small volume is added from the burette, and so on until a remarkably sharp end-point is reached, when the cells of the system become perfect biconcave disks. These operations should be carried out as quickly as possible; I think that there are time factors involved, but these require further study. Carrying out titrations in this way, we arrive at the results shown in Table 3, which gives the quantity Q of a number of substances required to produce disks from the spheres of a suspension made by adding 0·2 ml. of washed human red cells (5×10^9/ml.) to 1·8 ml. of lecithin sol, 50γ/ml. in concentration. This amount of sol contains about $0{\cdot}7 \times 10^{17}$ molecules.

Table 3

Reversing substance	Conc. mM./l.	Q ml.	No. of molecules	A.^{2} per molecule	Ratio
Benzene	10	0·6	36×10^{17}	4	51
Indol	5	0·6	18×10^{17}	8	25
Chloroform	25	0·2	30×10^{17}	5	43
Plasma	(0·4)	0·4	(10×10^{17})	(14)	(12)

In the fourth column of the table is shown the number of molecules of the reversing

substance contained in the amount Q which transforms the spheres into disks, and in the fifth column the number of molecules of reversing substance per A.^{2} of red-cell surface. It will be clear that when the reversal takes place there are about 10 times as many molecules present as would cover the surface, and also many more molecules of reversing substance than there are lecithin molecules (last column, headed 'ratio'). (The figures for plasma are based on the exceedingly doubtful assumption that the reversing substance in serum is serum albumin, and are added merely to show that the reversing effect of plasma is of the same order as that of benzene, indol, and chloroform.) Probably all the reversing substance does not gain access to the cell surface, a considerable proportion of it remaining in the suspension medium which separates individual cells and perhaps reacting with the lecithin there, but molecule for molecule, the amount of reversing substance needed to bring about the sphere-disk shape change is much greater than the amount of lecithin required for the original change from disk to sphere. Calculations of this kind are, of course, very approximate, but it seems that an order of 10 is involved in the ratio of molecules of reversing substance to molecules of lecithin needed for sphering, but not an order of 10^2.

4. THE STRUCTURE OF THE MEMBRANE AND THE MECHANISM OF SPHERING

Throughout this series of papers on the spherical form of the mammalian red cell I have avoided speculating on the mechanism which may be responsible for the shape transformations, because I have felt that the observations were not sufficiently quantitative and also because the phenomena themselves were insufficiently described. During the last five years, however, no essentially new shape changes have been observed, while a description of the transformations which occur in oval red cells (camels, birds, reptiles, amphibians, and fishes) has been added to the description of those which are observed in the mammalian biconcave disk; it therefore seems time to try to put the observations together so as to form some sort of picture of how the shape changes come about. Irrespective of how one approaches this problem, there are two points which are much clearer now than they were in 1929 when the first of this series of papers was written.

(1) 'The principal difficulty in dealing with the change in form lies in the fact that the shape which the mammalian red cell possesses when freely suspended in fluid itself requires explanation, whereas the spherical form corresponds to a condition of minimal surface energy and is to be expected; the problem of the disk-sphere transformation is thus intimately bound up with the problem of why the red cell is biconcave and discoidal instead of spherical, and can be approached in as many different ways as there are explanations for the discoidal shape' (Ponder, 1929). I have recently reviewed these explanations and the evidence for each of them (Ponder, 1941*a*), and of the four fundamental types of explanation (internal forces, internal structure, surface forces, and surface structure) the evidence is almost overwhelmingly in favour of the last, always with the proviso that the four points of view are not mutually exclusive, as is so often implied. Indeed, there is a good deal of evidence in favour of an internal structure (corresponding to Rollett's stroma) in addition to a surface structure, and the distinction between a surface ultrastructure and oriented surface forces is probably one of words except in so far as Norris, who originated the 'surface force' idea, sought to show that the biconcave form represents an equilibrium between *two simple* forces, one expansive,

and the other (surface tension) conducing to contraction (Norris, 1882; Ponder, 1933). It is almost certainly a mistake to seek to explain the shape of the red cell by a simple and purely physical explanation merely because the shape is simple and symmetrical, and we certainly do not look for any such simple explanation for the shape of other types of cell, such as the rod of Corti or the cone of the retina. We are content in these instances to say that the shape is determined by the molecular configuration as laid down during development, and it is sufficient to say the same thing for the erythrocyte, particularly when we consider that its antecedents are morphologically distinct (the reticulocyte with its network, the spherical nucleated normoblast, etc.) and that deviations from the normal course of development in the marrow result in great modification of the form (the ovalocyte, the poikilocyte, the 'target cell', etc.). The usual development results in the laying down of a molecular structure at the cell surface, and perhaps an internal structure as well, the practical distinction between the two being that evidence of the surface structure can be obtained by using polarized light (Schmitt, Bear & Ponder, 1936, 1938), whereas there is no such direct evidence of a structure situated internally. Granted that a surface ultrastructure exists as a result of whatever obscure forces determine the development of the biconcave disk, there is no difficulty in seeing that the total energy of the surface may be at a minimum although the surface is not a minimum for the enclosed volume, nor is there difficulty in seeing that the cell may become a sphere, with the smallest surface for the enclosed volume, if the orderly nature of the ultrastructure disappears, temporarily or permanently.*

(2) Because of the numbers of the molecules involved, the probability is very great that the disk-sphere transformations and their reversals brought about by such substances as lecithin, rose bengal, benzene, plasma, etc., are due to the action of these substances at the cell surface, and more specifically, between the molecules of these substances and the molecules of the surface ultrastructure.

These two points, I think, are abundantly clear on the evidence which has accumulated, and since the crux of the situation lies in the existence of a surface ultrastructure, we will now try to form a definite idea of what the structure is like.

(*a*) *Thickness*. The surface structure may be provisionally regarded as made up of a complex network of protein oriented tangentially and lipoid oriented radially. The principal lipoids are cephalin and cholesterol, the fatty acid chains of the former probably arranged side by side with the phosphoric acid and serine (Folch & Schneider, 1941) groups on the watery side of the interface. These groups seem to dominate the surface electrophoretically (Furchgott & Ponder, 1941). Such a picture will probably meet with general acceptance, for it incorporates the principal features of Danielli & Davson's (1935) model, and the results of the optical observations of Schmitt *et al.*

There will be less agreement about the total thickness of the structure, partly because the analytical figures given by different observers do not agree with each other, and partly because of an insistence in some quarters on the necessity of continuous molecular films of lipoid, protein, etc. Parpart & Dziemian's (1940) figures, which are probably the best of their kind, show that the amount of extractable lipoid, all of which is contained in the red-cell membrane as we know it, is not sufficient to make up more than a bimolecular

* A simple analogy will illustrate the idea. Consider a steel spring. Work has to be done either to extend it or to shorten it, and in its unstretched and unshortened state the free energy is at a minimum value. The fact that this minimal free energy corresponds to a certain length of the spring is determined by the structure of the metal and the arrangement of the molecules in it.

layer 30 A. thick. The protein moiety would supply layers with a total thickness of about 90 A., and so the thickness of the structure would add up to about 120 A. But Parpart & Dziemian point out that the lipoid may be bound to the protein (by means of the serine group of cephalin, perhaps) so that there may be an orientation of cephalin at particular loci around the protein molecules rather than a formation of a continuous bimolecular layer. (There is not enough cholesterol to form even a monolayer.) As judged by the action of lipases, the phospholipoids are present at the surface of the membrane (Ballantine & Parpart, 1940), and it is probably their phosphoric acid and serine groups which are responsible for the behaviour of the electrical mobility. But all the phospholipoid need not be at the surface; half of it, for instance, might form an oriented layer, although not a continuous monolayer, deep to an oriented layer of the other half, thus leaving some of the area of the surface of the membrane to be filled with protein molecules, and giving the surface a molecular mosaic structure.

Parpart & Dziemian's figure for the total thickness, 120 A., is about the same as that found by a direct gravimetric method (Fricke, Parker & Ponder, 1939), but it ought to be borne in mind that this is the thickness, exclusive of the contribution of water, and for the membrane of the ghost. Estimates of the extent to which water contributes to the thickness range from 10 to 100%; Waugh & Schmitt (1940) give about 25%, and their figures for total thickness, obtained by means of the leptoscope, are consistently higher than the foregoing: 135 A. at *p*H 7, 220 A. at *p*H 6, and even larger values. Even more recently (1941), Zwickau has obtained photographs of fixed and dried membranes of ghosts by means of the electron microscope, and sets the thickness (dry) at from 200 to 300 A. If the contribution of water is again allowed for, these values would be the highest yet suggested. Further, the thickness of the membrane in the intact cell may be greater than that in the ghost, for substances, perhaps not essential to resistance, capacity, or permeability, may diffuse away at the time of lysis; the anti-sphering substance (Furchgott & Ponder, 1940) is one such substance, and it makes up about one-third of the estimated protein content of the membrane of the disk. I therefore think that the thickness of the red cell membrane may approach 500 A., and so reach dimensions which are visible, if not resolvable, by the microscope. The importance of such a possibility, from our immediate point of view, lies in the relation between thickness and rigidity. For a uniform sheet, the rigidity increases approximately as the cube of the thickness, and so a membrane 300 A. thick, as suggested by Zwickau's measurements, would be 15 times as rigid as a similar membrane 120 A. thick.

(*b*) *The expansive forces.* The fatty acid chains of the radially oriented cephalin molecules are presumably bound together by weak Van der Waals forces, and we know nothing of the forces between, or the orientation of, the molecules of the protein moiety, but we can look for the 'expansive force' in the probable arrangement of the part of the cephalin molecules which sticks out from the surface into the watery side of the interface. The two fatty chains of each molecule, arranged in a zigzag along their length, are about 22 A. long and about 2·5 A. apart, while the central carbon of the glycerol (considering the β-form of cephalin meantime) is set at angle 109° to the carbons to which the chains are attached (Fig. 1). To this central carbon is attached the phosphoric acid, the OH group of which is strongly ionized with the H ion floating somewhere nearby in the water. To this is attached the serine, with its COOH group strongly ionized (ionization constant about 100 times that of acetic acid). We do not know at what angle

the phosphoric acid-serine chain is attached to the glycerol, but its length can be put at about 9 A., and there is no doubt but that it has two powerfully ionized acid groups, one from the phosphoric acid and one from the serine, as well as the terminal OH of the serine. These groups on adjacent oriented molecules being of the same sign, repulsive forces must exist between them, and these may be the 'expansive forces' which we refer to in connexion with the discoidal form, and which, on this view, would have their origin in the nature and orientation of the cephalin molecules.

(*c*) *The yield point.* The observation that the discoidal form shows a definite yield point has a bearing on the present problem which is best summarized by quoting from Houwink's excellent paper on 'The Yield Value' in the *Second Report on Viscosity and Plasticity* (1938). 'It is to be expected that in plastic deformation of highly polymerized

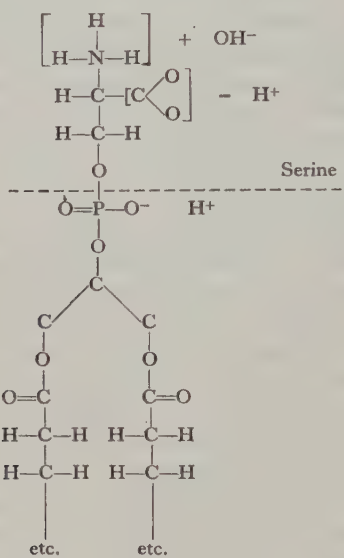

Fig. 1.

substances many of the weaker bonds are disrupted, so that the large molecules can be shifted with respect to each other without suffering great internal distortion. The large molecules thus function as a kind of nearly undistorted element in the process; they may be denoted as the kinetic units in the deformation. In particular, it may be surmised that when the units are sufficiently large, so that there are relatively great areas over which they are kept together by weaker bonds, stresses below a certain limit will be unable to cause a rupture of all these bonds, and so cannot produce flow; hence a more or less marked yield point is to be expected.' These conditions apply, qualitatively at least, to a structure such as we imagine the red cell membrane to be, the stress tending to produce deformation (towards the spherical form) presumably being the force of surface tension, and the resistance to it residing in the forces between oriented molecules.

(*d*) *The action of lecithin.* If the surface, dominated by mutually repulsive groups of cephalin, is brought into contact with a surface of a similar nature such as may quite

conceivably be formed by adding lecithin to the suspension medium, the repulsive forces in the surface would be weakened and might even vanish. The amount of lecithin needed to produce sphering has already been shown to be about enough to form a single lipoid layer at the cell surface, and as the molecules of the layer are likely to be oriented as a result of the orientation of the molecules in the membrane, the effect of adding lecithin to the suspension is to set up a surface system in which two opposed layers of lipoid molecules, one on the membrane side of the interface and the other on the watery side of the interface, are opposed to each other with their hydrophilic groups in contact. Under such circumstances the lateral repulsive forces between the members of the oriented layers would certainly diminish and might even disappear. The shell containing the hydrophilic groups of this coupled surface system, however, would be essentially the same as the shell of hydrophilic groups ordinarily present at the cell surface, and so the electrophoretic behaviour would not be expected to be much altered by the addition of lecithin.

(*e*) *The action of the reversing agents.* In general, the reversing agents which turn the lecithin sphere back into the disk are lipoid solvents. They therefore have an affinity for lecithin, and in the first instance for the lecithin which is responsible for the disk-sphere transformation and which forms the outer layer of the surface shell. It seems sufficient to suppose that the molecules of the reversing substances react with the oriented molecules in the outer part of the shell, take their place, and so permit the membrane molecules to return to their original (expanded, or discoidal) orientation. This first part of the effect is very similar to that of washing the added lecithin away from the cell surface. The molecules of the reversing substance, however, continue to show their affinity for lipoid by next reacting with the membrane molecules themselves, breaking down their orientation and producing prolytic spheres and ultimate haemolysis.* This second part of the effect is probably always reversible in its early stages, and an excellent instance of the reversibility is seen in the case of rose bengal, which produces a disk-sphere transformation which at first is easily reversed by the addition of plasma, but which later proceeds to the formation of prolytic spheres and lysis. In the case of most lysins, the early and the later stages of the second part of the effect are not so easily distinguished, if indeed they can be distinguished at all. Thus, when bile salts are added to lecithin spheres, the sequence of events is probably: first part of effect, replacement of lecithin molecules in outer part of shell by bile-salt molecules, with reassumption of the discoidal form; second part of effect, reaction of bile salt molecules, now occupying outer part of shell, with oriented cephalin molecules of membrane, disappearance of the orientation, appearance of the prolytic sphere, and eventual haemolysis. The second part of the effect is probably reversible in its early stages, but the reversibility is usually difficult to establish for technical reasons.

(*f*) *The influence of the interior, and 'permanent set'.* Anything like a complete picture

* In this discussion the principal emphasis has been placed on a union of haemolytic substances with lipoid components of the red-cell membrane, whereas in most of my investigations on the kinetics of haemolysis I have referred to the combination as principally one between the lysin and the cell proteins (Ponder, 1934). The latter point of view originated in the observation that plasma proteins combine with and inhibit the action of most lysins, and was originally put forward as an alternative to the now abandoned theory which explained the lytic action of bile salts, alcohols, etc., on the basis of a supposed *solvent* effect on the cell lipoids. The distinction between a reaction with protein and a reaction with lipoid is superfluous in view of our more modern conception of red cell membrane structure, for it would be virtually impossible to have a reaction with one type of component and not with the other.

of the mechanism of disk-sphere transformations must take two additional phenomena into account. The first of these is the extent to which the changes at the cell surface are modified by the presence of an internal structure. In the case of the orthochromatic mammalian erythrocyte, the internal structure appears to be so homogeneous and to have such a small rigidity that it is possible to treat the surface forces as if they were subject to no restraint. In the case of the mammalian poikilocyte, reticulocyte, and ovalocyte, and in the case of the nucleated red cells of the lower vertebrates, on the other hand, the factor which dominates the situation as regards shape transformations and the shape of the ghosts seems to be the rigidity and form of the internal structure.

The second phenomenon involves the fact that ghosts are unable to undergo disk-sphere transformations (Ponder, 1942). After haemolysis by water, the ghost rapidly assumes the biconcave shape and the volume of the cell from which it was derived, but no sphering can be observed between slide and slip, with lecithin, with saponin even in concentrations as great as 10%, with bile salts, or with rose bengal. The molecules of the surface membrane, distorted in the stretched prolytic sphere, return to their original positions when the stretching forces disappear at the moment of lysis, after which, the original orientation re-established, the surface structure seems to undergo something analogous to 'permanent set' in its biconcave form. It may seem rather far-fetched to speak of the biconcave form of the surface structure as its extended or β-form, and of the spherical form as an α-form, but an analogy with the extended and contracted forms of the keratin grids of wool quite suitably expresses the general idea of the relation of red cell shape to the configuration and orientation of the surface molecules.

SUMMARY

1. The disk-sphere transformation produced in the mammalian red cell by lecithin can be studied quantitatively if the lecithin is added to the cells in the form of an isotonic sol, and if the preparations are examined between plastic slides and cover-slips.

2. The quantity of lecithin required to produce spheres from disks is approximately the same as that needed to form a monolayer at the red-cell surfaces.

3. The temperature coefficient of the lecithin disk-sphere transformation is small and positive.

4. The red-cell structure possesses a definite yield point, a certain quantity of lecithin being required to initiate the shape changes. Such yield points are characteristic of structures made up of large oriented molecules. The effect of fixation is to increase the yield point, the increase being a function of the concentration of the fixative.

5. The lecithin disk-sphere transformation can be reversed by the addition of lipoid solvents such as benzene, indol, chloroform, etc. The effect is a strictly quantitative one, the number of molecules required being about 10 times as great as that required to cover the cell surfaces, and somewhat more than 10 times as great as the number of lecithin molecules needed to bring about the disk-sphere transformation. The reversal of the transformation is usually followed by haemolysis, as most of the reversing substances are lysins.

6. The red-cell membrane is probably an ultrastructure of oriented molecules, about 200–500 A. thick, and seems to be able to exist in two metastable forms, the extended or discoidal form, and the contracted or spherical form. It is suggested that the seat of the

forces which produce the extended form lies in the repulsions between the ionized hydrophilic groups of oriented cephalin molecules at the cell surface, and that the effect of a lecithin concentration at the surface is to diminish or abolish these forces. Reversing agents combine with the lecithin, and thus leave the repulsive forces able to establish the discoidal form again, at least temporarily.

7. Attention is called to the influence of the red-cell interior as modifying the effects of these essentially surface forces, particularly in reticulocytes, poikilocytes, and the nucleated red cells of the lower vertebrates.

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THE BLOOD PRESSURE OF THE CHICK EMBRYO DURING DEVELOPMENT

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(With 4 Text-figures)

INTRODUCTION

This investigation was made as part of a wider inquiry into the developmental physiology of chick circulation in relation to the histology of the developing vascular system.

One observation on the blood pressure of the chick embryo has already been made by Hill & Azuma (1927) who measured the pressure in the arteries of the chick blastoderm after 2 days' incubation and found it to be $2\frac{1}{2}$ cm. of water.

Their method, like that used in the present work, is a sphygmomanometric one. To the blastoderm freed from its surroundings they applied an external air pressure, transmitted through an elastic transparent membrane thereby compressing the blastoderm against a glass plate; the least external pressure necessary to collapse the arteries was then measured.

Hill & Azuma's method as it stands cannot be readily applied to later stages of incubation because of the difficulty of freeing the larger area vasculosa of older embryos from yolk and white without injury.

The method described below has been developed for measurements on the arteries of the chorio-allantois and does not involve interference with the yolk sac. It can only be used, however, when the chorio-allantois has reached a certain size which prohibits measurements earlier than the sixth day of incubation.

METHODS

Thirty-three successful measurements have been made on embryos of 6–19 days' incubation, the chorio-allantoic arteries ranging in diameter from 0·25 to 0·75 mm. The final curve of arterial pressure during development (Fig. 4) includes the value for the 2-day chick obtained by Hill & Azuma.

The artery in which the blood pressure is to be measured is compressed between a glass probe, inserted through an incision in the vascular membrane, and a capsule, 2 cm. in diameter, to which air is admitted under pressure and of which the floor is formed by a membrane of thin rubber with a hole in it (Fig. 1). This hole is smaller in diameter than the glass probe. The hole and probe are placed concentrically when measurements are made, and the vertical distance between them is adjusted with great care. If they are too close, the artery is compressed without any air pressure being applied

to the chamber, but if they are too far apart, bubbles of air escape.* The artery is compressed directly by the air in the chamber.

If a continuous rubber membrane was employed, any curvature imparted to it would introduce an error into the measurements, for the pressure on the two sides of the membrane would not be the same, and it would be almost impossible to use the device with

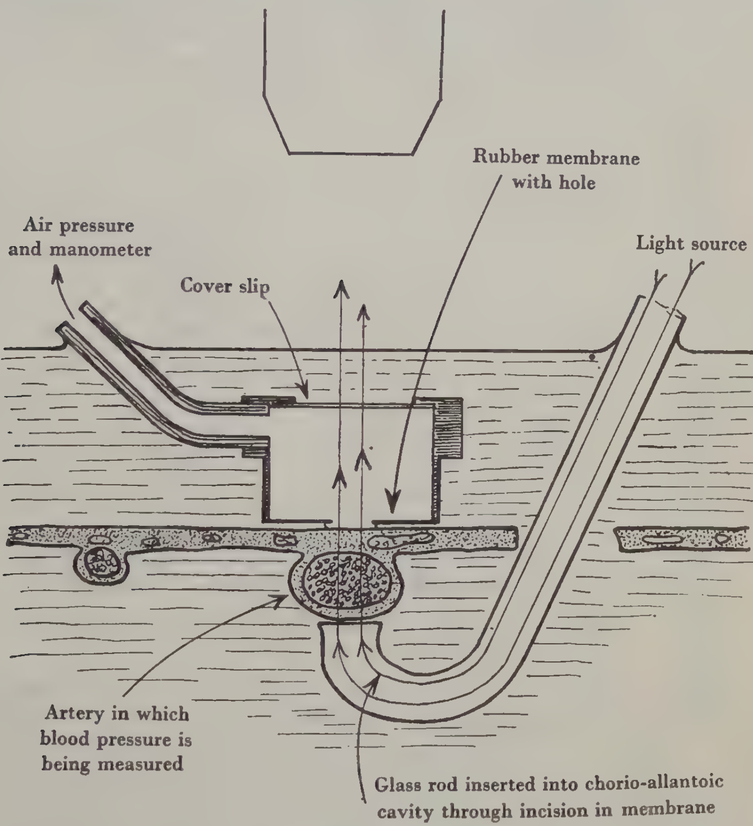

Fig. 1. Apparatus for measurement of arterial pressure in chorio-allantoic artery of chick.

no curvature of the membrane. The errors thus introduced would be serious as the pressures to be measured are small in comparison with those usually measured by sphygmomanometric methods. The earliest trials of this apparatus were made with continuous membranes and although the thinnest rubber sheeting obtainable was used, no uniform readings could be obtained.

The probe and the brass tube attached to the capsule are both carried by special micro-manipulator heads, which give a coarse movement over a wide range in all three

* In practice, the distance between hole and probe is kept as large as is possible without bubbles of air escaping. The error in the result due to the pressure inside the air bubble is less than 0·5 cm. of water.

dimensions. In the upper end of the glass probe a 6 V. 'flash-lamp' bulb is mounted. The probe thus serves as a glass rod illuminator, and the artery is clearly seen under a low-power microscope through the hole in the rubber membrane, and the cover-slip forming the roof of the pressure capsule. The inside of this cover-slip needs moistening with glycerine to prevent condensation.

The vessel containing the saline, in which egg and device are immersed, has a capacity of about 250 c.c. and is heated by a compact form of water-bath, controlled to give the necessary temperature of 38–39° C.

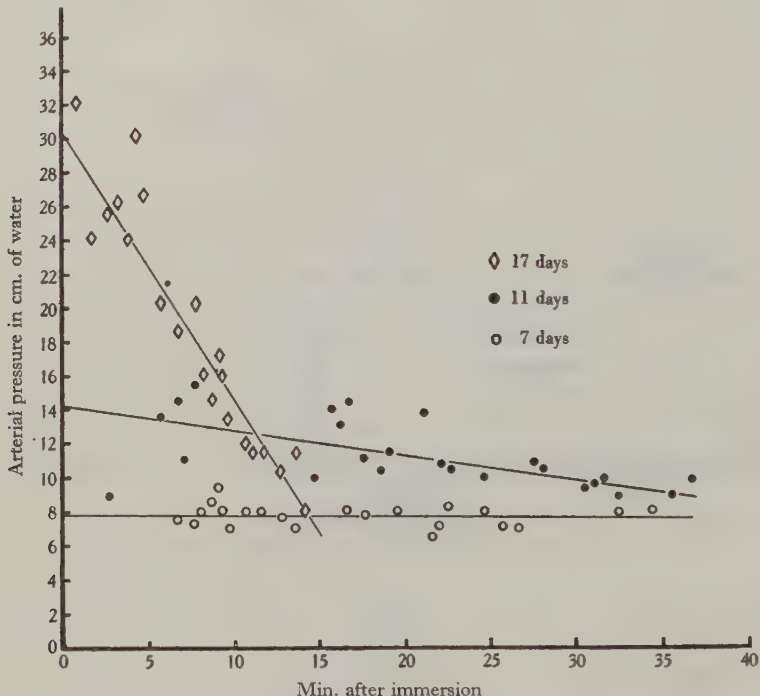

Fig. 2. Arterial pressure observations during the progress of an individual experiment.

The procedure is as follows. A hole is made in the air space of the egg which is then immersed in the warm saline, and with extreme care the shell is gradually picked off, using an outward motion. When most of the shell is removed, the shell membrane is peeled off, with even greater care. The last third of shell and shell membrane can usually be floated away from their contents. The whole chorio-allantoic membrane is then exposed, covering nearly all the yolk sac and albumen. In stages up to 12 days or so, it is advisable to free the chorio-allantois from the yolk sac by careful tearing at the edge of the former where the two embryonic membranes are attached to each other. When the operation is successful, a preparation can be made of the embryo in its amnion with yolk sac and chorio-allantois intact on each side.

Next, an accessible chorio-allantoic artery is selected, and a small incision made in the membrane near it, through which the probe is inserted. The capsule is cautiously lowered, and the artery made to lie suitably across the device, by gently pulling on the

membrane. The probe and membrane are brought near together to compress the artery, and air pressure is admitted to the capsule. Probe and membrane are now separated until air bubbles are on the point of escaping from the hole in the membrane, and the air pressure is varied until the artery can be compressed by this agency alone. The pressure is then adjusted until a point midway between systolic and diastolic pressure is found, so that the blood pulsates across the hole from the side nearest the heart with each heart beat, being squeezed out during diastole. Separate measurements for systolic and diastolic pressures are not attempted, as only in this intermediate position is a clear end-point obtainable. The air pressures applied are measured on a water manometer, and pressure and time of observation are noted down over a period of 10–30 min. The arterial pressure

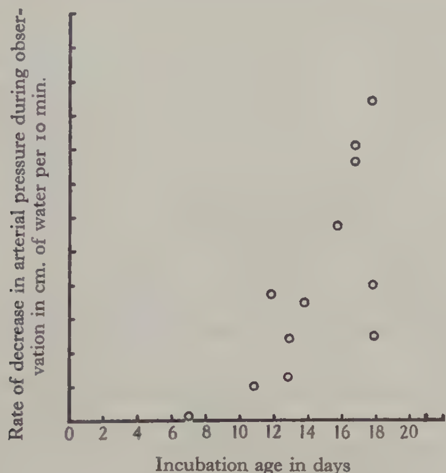

Fig. 3. Average rate of fall of arterial pressure during an individual experiment related to the age of the embryo.

gradually falls as the preparation deteriorates (Fig. 2). Up to the tenth day of incubation, the fall in arterial pressure with time under observation is very gradual. This is due to the extreme ease with which eggs of this age can be decanted, as described above, with almost no haemorrhage and with the chorio-allantoic circulation almost unaffected. In the second half of the incubation period, however, increasingly more damage is done by this treatment. Since the chorio-allantoic capillaries go into stasis with the slightest mechanical stimulus and haemorrhage is unavoidable with any manipulation at all, conditions for the measurements of the arterial pressure become less favourable, as the eggs become older. This is reflected in the curves of Fig. 2 in which the arterial pressure in embryos of different ages is plotted against time of observation; the curve at 17 days is much steeper than at earlier stages. Fig. 3 expresses the slope of these curves for twelve sets of observations, and it is seen that they usually, though not always, become much steeper in later stages.

The question thus arises as to how our final estimate of the arterial pressure at each stage is to be made. Up to 10 days of incubation, a simple average of all values obtained in each set of observations is clearly sufficient, but in later stages this gives merely an average value of the arterial pressure of embryos dying from anoxemia. It seems more

reasonable to plot at each stage the curve of decrease in arterial pressure with time of observation where the constituent points can obviously be represented by a straight line and to read off from this line the estimated arterial pressure at the beginning of observation. In Fig. 4 the circles express these estimated arterial pressures, and to them most weight has been given in drawing the final curve of arterial pressure against incubation time. The solid points in Fig. 4 represent the averaged values, and where estimated and averaged values are given for the same set of observations, the two points are joined by a vertical line. These lines express the divergence between averaged and estimated arterial pressures.

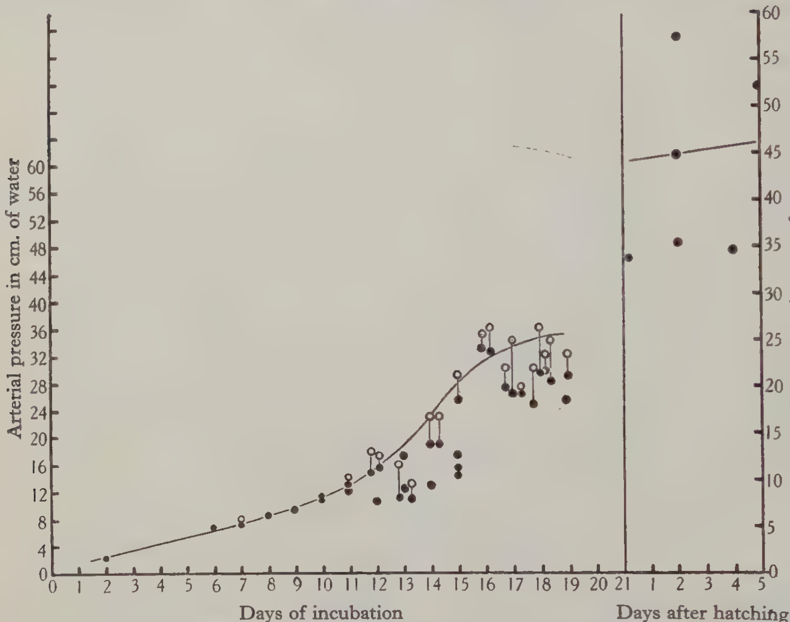

Fig. 4. Arterial pressure of chick embryo and chick respectively in respect of age. ● = average values for a given determination. ○ = value calculated from extrapolation of individual observation (see Fig. 2).

The arterial pressure in newly hatched chicks was measured by a similar method, except that a continuous rubber membrane and a mercury manometer were used. The chicks were anaesthetized with ether, and the common carotid arteries in the neck were exposed, an illuminated glass spatula was inserted between the arteries and neck muscles, and the capsule applied to the upper surface of the arteries. Six sets of observations were made on chicks in the first week after hatching and others at later stages. The slope of the curve for stages after hatching in Fig. 4 is taken from these observations.

DISCUSSION OF RESULTS

It is proposed to discuss these results at length in a subsequent paper, together with other data from the embryonic circulation. Meanwhile the data on arterial pressure in the chick embryo can be compared with the corresponding results from mammalian foetuses which have been obtained by various authors, notably by Barcroft (1935, 1936).

Arterial pressure for the sheep (Barcroft, 1936) plotted against foetal age gives a curve of the same general shape as mine for the chick embryo, rising, at first gradually, then progressively more steeply. The time scales are respectively 150 and 21 days. It is interesting to compare the sheep and chick embryos when their size and arterial pressure are about the same. The sheep foetus at 44 days and the chick embryos at $14\frac{1}{2}$ days both have the same arterial pressure (18 mm. mercury) and weight (10 g., Needham, 1931). The arterial pressure of the chick embryo is rising very rapidly at that stage, while that of the sheep embryo is still in the initial slow rise. The chick embryo also is developmentally much further advanced than the sheep embryo of the same weight.

A further resemblance can be seen between the curves for arterial pressure during development in chick and sheep embryos. In both the rapid rise in arterial pressure in the latter half of development is followed by a period of stationary pressure just before birth. Barcroft gives the arterial pressure of the sheep foetus at 140 days—10 days before term—at 76 mm. of mercury; in the continuous tracing of arterial pressure during birth by Caesarian section given in the 1935 paper, the pressure at first is 63 mm. of mercury. In the chick embryo the pressure rises very little after 16 days.

Again, at birth, when pulmonary respiration is established, the arterial pressure in both animals rises to a figure which continues the preceding rapid rise, interrupted towards the end of embryonic life. In the sheep foetus the rise in arterial pressure at birth is about 20%, but in the chick embryo the rise appears to be larger and in the data of Fig. 4 lies between 34 and 100%.

The stationary period in arterial pressure at the end of development in the chick embryo is a very interesting problem in developmental physiology. The reason for it certainly does not lie in the heart itself, the weight of which continues to increase at the end of development as rapidly as before and doubles between 16 and 21 days (Olivo, 1930).

On opening an egg at the end of the third week of incubation, one is struck by the extreme darkness of the blood in the chorio-allantoic vessels. The blood in the arteries appears fully reduced and that in the veins far from saturation with oxygen. Unfortunately, no measurements appear to have been made on the oxygen content of the blood of the chick embryo at this stage, a problem which becomes all the more interesting in view of the investigations on mammalian foetuses by Barcroft and co-workers. Appearances, however, suggest that the chick embryo at the end of development is in a state of anoxaemia so far advanced that the action of the heart may be adversely affected.

SUMMARY

1. An apparatus is described for the measurement of the pressure in the chorio-allantoic artery of the chick embryo.

2. Data are given of such measurements ranging in time from the second to the nineteenth days of incubation.

3. Some measurements are also given of the arterial pressure of the chick within the first five days after incubation.

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NARCOSIS AND ASPHYXIATION IN SOME SPECIES AND MUTANTS OF *DROSOPHILA*

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(With Eight Text-figures)

I. INTRODUCTION

This paper is concerned with (1) the relationship between narcosis and asphyxiation, (2) the physiological analysis of the insect organism, (3) the influence on recovery from narcosis or asphyxiation of age, *p*H of food, etc., and (4) the differences in narcosis in *Drosophila* species, subspecies and mutants.

(1) Different physiological schools seek to explain the effects of narcosis in various ways, such as asphyxiation, solution of the narcotic in lipoids, its absorption or permeation, etc. In this paper some similarities and differences between asphyxiation and narcosis are described, while experiments dealing with their interaction are discussed.

(2) Narcotization is an excellent method for the physiological analysis of organisms, as was first emphasized by Claude Bernard (1875). The trachealization of the central nervous system in insects is especially favourable for study, since the circulatory system does not interfere as it does in vertebrates. Volatile narcotics can pass almost directly from the atmosphere to the nervous tissue, as can oxygen after asphyxiation (Kalmus, 1935).

In this paper I attempt to explain some of the results obtained on the assumption that macrophases of different lipoid and water content exist in the fly.

(3) The influence of age on the resistance of an individual to oxygen lack is well known for mammals (e.g. Reiss & Haurowitz, 1929). Here I show that young insect imagines remain active with a lower oxygen supply than adult individuals, and that the recovery curves in etherizing experiments differ for newly hatched and older *Drosophila* flies. In addition to the age of the individual fly, the age of the culture bottle must be taken into consideration as a factor influencing narcosis. An attempt has been made to imitate the influence on narcosis of changes in the food medium by varying the *p*H of special media.

(4) In a laboratory where many stocks of *Drosophila* are kept and studied, this material provides an opportunity of trying to establish differences in narcotizability between species, subspecies and mutant races. Such differences have been established (Sekla, 1937), and are here investigated.

II. CRITERIA FOR THE DEGREE OF ASPHYXIATION OR NARCOTIZATION. RECOVERY TIME

Narcosis and suffocation, although quite different in their action on vertebrates, show striking similarities in insects. Reduction of oxygen below a certain level or increase of concentration of a narcotic (ether, chloroform, carbon dioxide) above a certain level,

both result in an immobilization which can be maintained for some time, and reversed by bringing back the concentration to that of normal air. However, there are certain differences in behaviour at the beginning of both processes. A gradual decrease of oxygen does not cause initial excitation corresponding to the dyspnoea of a mammal, but leads to a decreased mobility, and gradually to complete immobilization (Csik, 1939). On the other hand, etherization or treatment with chloroform or carbon dioxide provokes a very marked initial excitation. Near the limiting concentration this excitation stage may last for a considerable time before sudden immobilization occurs, and several phases of unco-ordinated and violent fluttering may alternate with immobilization. Similar but less well-marked behaviour may occasionally be observed during recovery from heavier narcosis; it is absent during recovery from oxygen want.

Asphyxiation supposedly causes immobilization by greatly reducing the production of nervous impulses and muscular energy. It may be caused by removal of oxygen from the air by means of a vacuum or an inert gas, or by poisoning the respiratory ferment by carbon monoxide or hydrocyanic acid gas. The action of the former on insects has been investigated by Haldane (1927), and that of the latter on *Drosophila* by Bliss (1935) and Broadbent & Bliss (1936). Their results will be considered later.

During narcosis the narcotic (ether, chloroform, carbon dioxide, etc.) accumulates on the loci where nervous excitation is produced, i.e. on structures within, or on the surface of, the nervous cells, and it is assumed that its specific action is the prevention of the permeation of those ions, on which the primary process of excitation depends.

The blocking of the cell surfaces becomes effective when the accumulation reaches a concentration specific for different narcotics; this concentration, the 'local limiting concentration', cannot be determined directly. It is, however, characterized by the external limiting concentration in the gas. The local limiting concentration is below a concentration to be called the 'local saturation concentration'.

Three theoretical criteria are available for the study of narcosis.

(1) The *stupefaction time* can be measured, i.e. the time from the beginning of narcotization to the moment when co-ordinated locomotion ceases. As it is difficult to time the cessation of movement of many flies included in a glass vessel, stupefaction time was measured only occasionally.

(2) The *minimum concentration* of the narcotic *in the air* necessary to immobilize the flies when permanently applied can be determined. Only approximate values can be obtained, mainly for the following two reasons: (*a*) The production of gas mixtures containing volatile anaesthetics of defined concentration is difficult. (*b*) There is no sharp change in the behaviour of a fly in the neighbourhood of this concentration, and apparently no stable equilibrium is ever reached between the concentrations of the phases of body and air. Similarly, in a *Drosophila* species the maximum concentration of oxygen causing immobilization varies within a wide range, between 1 and 4% (Csik, 1939).

(3) The most exact and easily determinable criterion is the *recovery time*, i.e. the period from the end of narcotization to the moment when a defined co-ordinated reaction-movement towards the light, either spontaneous or after stimulation—is regained by the fly.

In a narcosis experiment two steps follow one another:

(1) During the 'influx period' the flies are kept in the narcotizing vapour or gas, the narcotic flows in and reaches certain concentrations at different places in the body. The

first part of this phase is the 'stupefaction period' lasting from the start of the experiment till the moment of complete immobilization, when the local limiting concentration is reached. The second part is the super-narcotization period.

(2) During the 'efflux period' the narcotic flows out of the body, now kept in air or specially prepared gas mixtures, till its concentration reaches zero. Generally only the first part of this phase is observed, i.e. the period till the defined locomotor reaction is regained, and it is called 'recovery time'. The 'remainder' of the efflux period is important when flies are repeatedly used in experiments (p. 251), and it must be remembered that it is not identical with the time necessary for recuperation.

III. METHODS

Two different techniques were adopted according to the nature of immobilization:

(A) *Gases and vacuum.* Selected batches of six to thirty flies were enclosed in glass tubes between cotton-wool stoppers and subjected for a few seconds to several hours to a stream of single gases, gas mixtures, or evacuated by means of a pump. The pressure of the gas as well as the speed of the pump were kept fairly constant. The following figures are taken from a carbon dioxide experiment (Table 1).

Table 1. *Recovery time from 25 sec. exposure to concentrated carbon dioxide. Three subsequent treatments of six flies* (Drosophila melanogaster or +). 4 *October* 1941. $T=21°$ C.

Individual	1st narcosis sec.	2nd narcosis sec.	3rd narcosis sec.	Total sec.
1st ♀	104	93	95	292
2nd ♀	93	100	99	292
3rd ♀	86	101	93	280
1st ♂	81	91	110	282
2nd ♂	108	90	88	286
3rd ♂	106	88	104	298
Total	578	563	589	1730

Statistical treatment of these figures shows that the differences, both between individual flies and different narcoses, are well below the expected values, and it is clear that the method is quite reliable.

(B) *Ether and chloroform vapours.* Equal batches of flies were transferred from a vial to a broad-necked jar of 335 c.c. capacity, and the jar closed and shaken. Ether or chloroform had previously been pipetted into the jar by means of a pipette graduated in hundredths of c.c., and the jar closed and shaken. After a measured time the flies were removed from the jar to a piece of paper, selected in groups, and the individual recovery times were noted with a stop-watch.

The goodness of the experimental method, as shown by repetition, is demonstrated on p. 241 (Table 2).

Statistical treatment of these figures shows that the differences between narcoses are not significant. But there is some indication of real differences in recovery of different individuals.

In experiments using very low or very high dosages of narcotic it is impossible to determine mean recovery times, as in the first instance some flies are not immobilized at all, whereas in the latter some flies die. However, the recovery of half the individuals

Table 2. *Recovery time of six* Drosophila simulans *from* $1\frac{1}{2}$ *min. exposure to air containing* 12·75 *vol.* % *ether.* $T = 21 \cdot 5°$ C. *The second treatment took place* 30 *min. after the first. The third treatment* $1\frac{1}{2}$ *hr. later.*

Individual	1st narcosis min.	2nd narcosis min.	3rd narcosis min.	Mean min.
1st ♀	9·0	10·1	9·8	9·6
2nd ♀	10·1	7·2	8·3	8·5
3rd ♀	10·9	8·8	10·9	10·2
1st ♂	11·0	10·3	11·8	11·0
2nd ♂	8·2	9·2	9·2	8·9
3rd ♂	7·6	7·9	10·2	8·6
Mean	9·2	8·9	10·0	9·5

can still be timed exactly under these extreme conditions. Therefore the *median recovery time* was used in most experiments. At medium concentrations there is very little difference between the mean and the median.

IV. RECOVERY FROM ASPHYXIATION

Recovery times after removal of oxygen for a measured time did not differ greatly, either for a vacuum or an indifferent gas (nitrogen, hydrogen). As the technique is easier, the results of exposure to hydrogen are the most regular. The results of an experiment of this type performed on *D. melanogaster or* + are shown in Fig. 1.

By plotting the logarithms of the asphyxiation time (a) against median recovery time (r) a straight line is obtained of the formula

$$r = 184 \cdot 5 \log a - 147 \cdot 6. \tag{1}$$

According to this formula recovery would be instantaneous after exposure to hydrogen for 6·3 sec. The formula indicates that after a short period of asphyxiation most of the oxygen stored in the flies' tissues has been consumed, and thus a stationary stage is reached where no more oxygen can be removed; the recovery from this state remains fairly constant for a considerable time.

V. RECOVERY FROM CARBON DIOXIDE NARCOSIS

By plotting the logarithms of the influx time (a) of carbon dioxide against median recovery time (r) a straight line similar to that for hydrogen is obtained; its formula is

$$r = 62 \cdot 5 \log a - 25 \cdot 0. \tag{2}$$

According to this equation recovery would be instantaneous after exposure to carbon dioxide for 2·5 sec.

Fig. 1 shows that the carbon dioxide recovery curve is less steep than the hydrogen curve. Both cross at an exposure period of about 10 sec. where there is little difference in recovery time. After exposure for less than 10 sec. flies treated with hydrogen recover quicker, after exposure for more than 10 sec. carbon dioxide-treated flies start moving first.

The exponential relation between recovery time and time of exposure to hydrogen can be explained by assuming that an asphyxial metabolite (perhaps lactic acid) is formed at a steady rate during exposure to hydrogen, and this immobilizes the fly when it reaches

a certain threshold. The substance is destroyed at a rate proportional to its concentration when the fly is replaced in air; similarly carbon dioxide diffuses in at an almost constant rate and diffuses out at a rate proportional to its concentration.

VI. EFFECT OF NITROGEN AND HIGH AIR PRESSURE

The rate of recovery after exposure of 100% nitrogen at normal pressure was much the same as after treatment with hydrogen. In pressure experiments performed on human beings, including myself (Case & Haldane, 1941), I observed that at 10 atm. air pressure

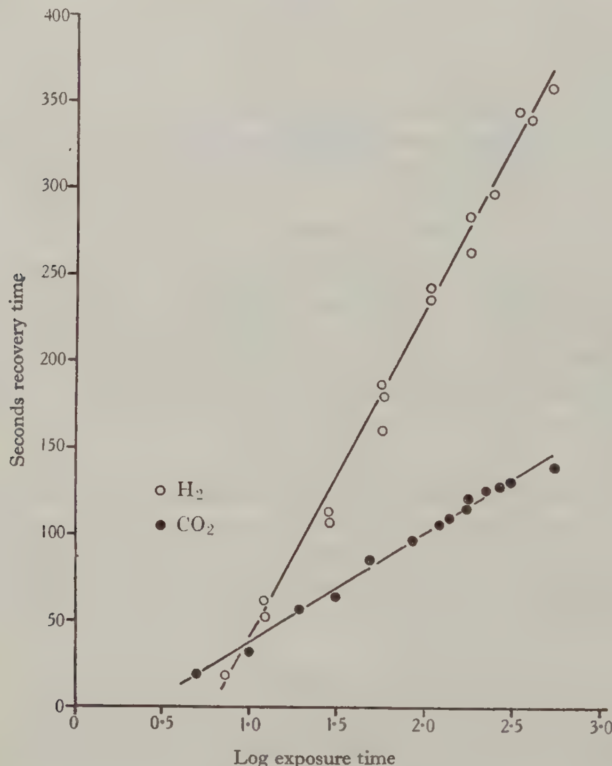

Fig. 1. Median recovery time plotted against log of median exposure time. *D. melanogaster* ♀♀. Hydrogen and carbon dioxide.

Drosophila flies became very lethargic and did not fly even when stimulated. This effect corresponds to the narcotizing effect on human beings under the same conditions, where it is generally ascribed to the action of the 8 atm. nitrogen (Behnke, Thomson & Motley, 1935).

VII. RECOVERY FROM ETHER NARCOSIS

Recovery curves after ether narcosis vary considerably according to the material used. The only general statement which can be made is that any increase in ether dosage, either by increasing influx time or concentration, increases recovery time. Two methods have

been selected to demonstrate the results obtained: (*a*) isonarcotic curves, (*b*) curves plotting dosage against recovery time.

(*a*) *Isonarcotic curves*

The same recovery time can be obtained experimentally with the influx of a high narcosis concentration for a short period as with a lower concentration effective for a longer time. Curves connecting points of equal recovery time may be called isonarcotic curves. A number of them is shown in Fig. 2. They are of the same type as the curves for survival from exposure to poisonous gases given by Flury & Zernik (1931).

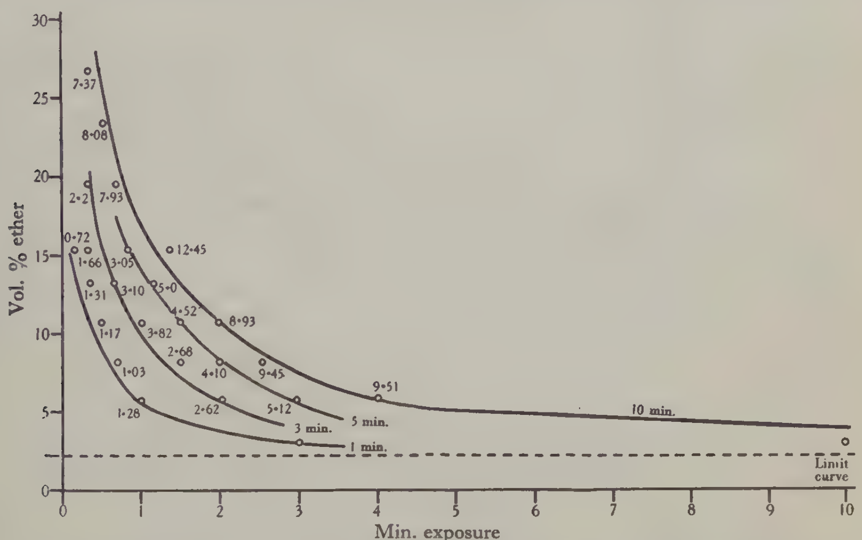

Fig. 2. Isonarcotic curves for equal duration of ether narcosis. *D. melanogaster or* + ♀♀.

(*b*) *Dosage-recovery curves*

Fig. 3 shows the median recovery times of batches of *D. pseudoobscura* plotted against ether dosages. The dosage is calculated as the product of the difference between actual and limiting (2·4 vol. %) ether concentration and time. Recovery time in this species increases in a wide range in direct proportion to the ether dosage. The formula is

$$r = 0{\cdot}73a\,(c - 2{\cdot}4) - 2{\cdot}91. \tag{3}$$

This formula again resembles Flury's and Zernik's formula (p. 100) for the action of poisonous gases. Similar results were obtained with *D. virilis*, but rarely with *D. subobscura*. Thus it appears that there is a fundamental difference between the shape of recovery curves from asphyxiation or carbon dioxide intoxication and etherization. The former show a logarithmic relation between dosage and recovery time, the latter a linear relation. However, in other species (e.g. *melanogaster*, *simulans*, and sometimes *subobscura*) or under different conditions, curves of quite a different type are obtained after etherization, some being logarithmic, others showing a point of inflexion.

This behaviour can be explained on two assumptions:

(1) Narcosis takes place so long as the ether concentration on the primary loci of stimulation, i.e. an aqueous phase, is above a certain level.

(2) Ether can pass into and out of this aqueous phase, not only into the outside air but into a lipoid phase inside the animal. During narcotization ether passes from the air mainly through the aqueous phase, into the lipoid phase, where the presence of ether will prolong recovery only after the partial pressure of ether in it is equivalent to that needed for narcosis in the aqueous phase.

The lipoid phase can be large or small in quantity relative to the aqueous phase, and the exchange of ether between it and the air can take place to a different degree through

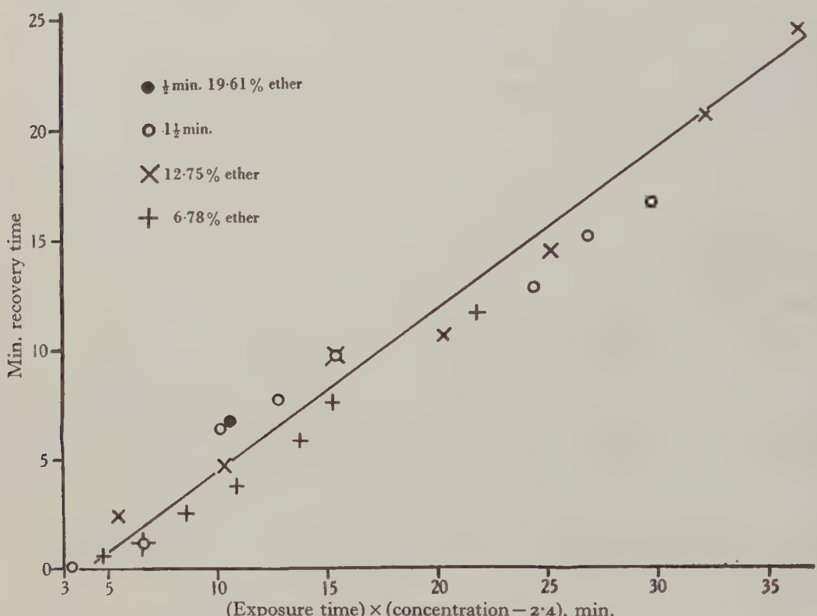

Fig. 3. Product of (median exposure time) and (ether concentration minus 2·4) plotted against median recovery time. *D. pseudoobscura* ♀♀.

the aqueous phase or directly. By varying one or both of these factors most of the types of the curves observed can be explained.

Figs. 4 and 5 show some examples of simpler curves obtained by plotting recovery time against vapour concentration at constant influx periods or against influx time at constant ether concentration.

Both figures show that the recovery curves of *D. melanogaster or* + males after small ether dosages (short time or low concentration) are sigmoid; large ether dosages give recovery times the curve of which corresponds only to the upper part of the sigmoid, as the point of inflexion is very near the *X*-axis, or even below it. This latter type of curve resembles somewhat the logarithmic curves obtained with hydrogen or carbon dioxide. However, there is one difference; if the dosage is increased still further the curve rises again steeply and becomes almost vertical, i.e. after very heavy ether dosages

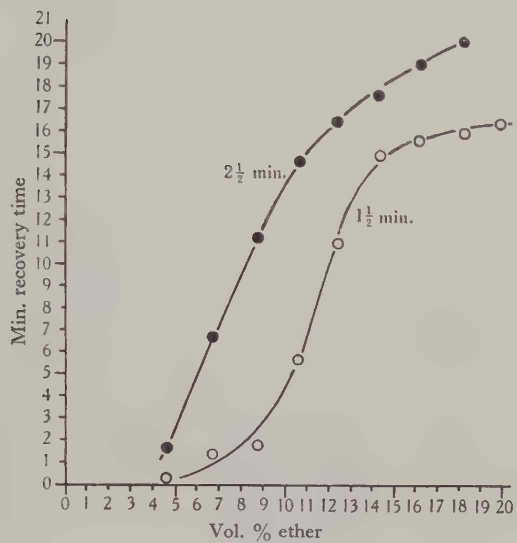

Fig. 4. Median recovery time plotted against ether concentration. *D. melanogaster or* + ♀♀.

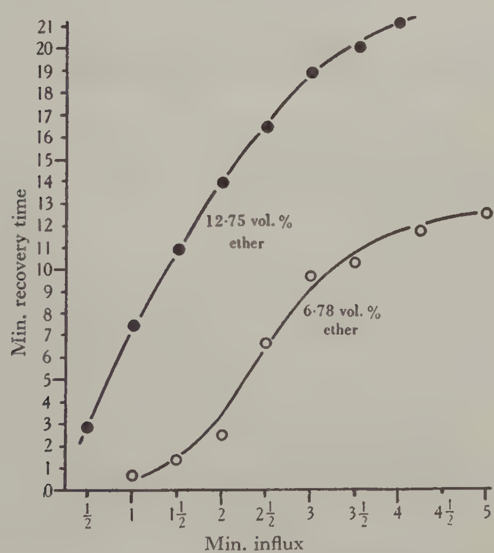

Fig. 5. Median recovery time plotted against median etherization time. *D. melanogaster or* + ♂♂.

flies do not recover at all (Fig. 6*a*). This is probably due to an irreversible process which is quite independent of narcosis. Now it is easy to imagine that this ether poisoning sometimes starts at dosages where the horizontal branch of the recovery curve is not yet reached. The result (Fig. 6*b*) is a curve which immediately gets steeper instead of decreasing its slope. As the upper ends of such curves do not depict narcosis, we need not discuss them further.

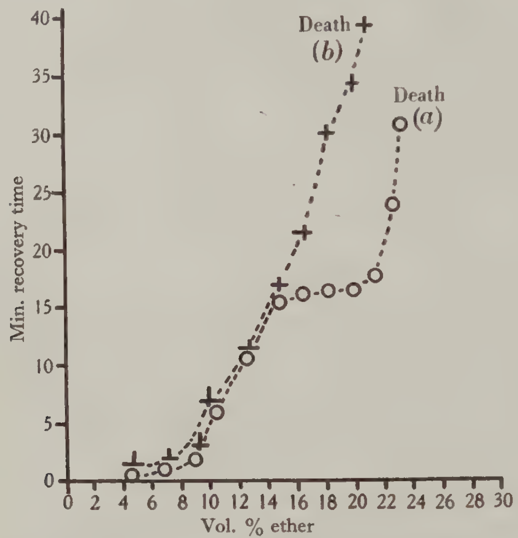

Fig. 6. Two different batches of *D. melanogaster* or + ♂♂ showing different resistance to heavy ether concentrations; 1½ min. etherization, median recovery times.

VIII. DIFFERENCES IN RECOVERY TIME DUE TO SEX AND AGE

The wide range of variation usually observed in the recovery times of individuals from one culture as well as the differences of recovery curves, can be markedly reduced by the control of several factors.

The size of the individuals of a species does not seem to have much influence on recovery time. More important are the sex and age of the flies. After narcosis with low and medium ether dosages, females generally recover more quickly than males from the same bottle. After application of high concentrations this behaviour is reversed. In *D. melanogaster* and *simulans*, where morphological sexual differences are most apparent, the difference in recovery also seems to be most marked. In the other species observed (*subobscura*, *funebris*, *pseudoobscura*, *miranda*, *virilis*, *montium*) it is smaller, but still significant. The difference of recovery between the sexes at low and medium concentrations may be a little greater than stated by this method, as females run much more slowly than males, and therefore may be considered as-being sluggish for a longer period. A slight difference, possibly significant, in the mean recovery times between virgin and mated females in *D. melanogaster* was observed, the virgins recovering more slowly.

The influence of age on the recovery time is rather complex. There is no doubt that fundamental changes in ether resistance occur during the first hours after emergence

(in *D. virilis*, during the first few days). After the application of low and medium concentrations the very young flies recover much earlier than the older ones, and frequently they do not become narcotized at all. At high concentrations the opposite behaviour may be observed, the young flies remaining narcotized several times longer than the older control animals. One possible explanation of this behaviour is that parts of the tracheal system are still filled with liquid for some hours after emergence, so that the ether penetrates more slowly into the central nervous system; but once in it would also take longer to get out again.

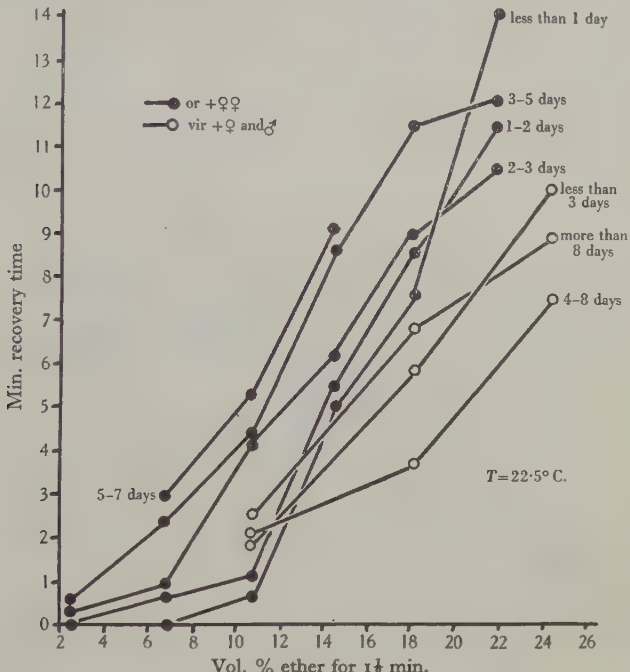

Fig. 7. Recovery times of *D. melanogaster or* + and *D. virilis* + of different ages.

After these changes during the first few hours of life, which show considerable individual variation (see also p. 240), a slight lengthening of the period of recovery can be observed for several days (Fig. 7), where the lengthening of recovery time at high dosages of flies less than 1 day old (3 days in *D. virilis*) can still be noticed.

IX. THE *p*H OF THE CULTURE MEDIUM AS A CONTROLLING FACTOR

It was found that older flies (10 or more days old) sometimes recover more quickly at medium ether concentrations than do medium-aged flies, and that different results were obtained when flies from bottles of different ages or from bottles and vials were compared. Also the first comparative measurements of species and mutants often gave contradictory results. This was explained by the fact that the 'age' of the culture influences recovery time independently of the age of the individual flies. Later it appeared that, apart from variations in the amount of food and moisture available, the actual reaction of the medium on which the flies are kept is the main factor responsible for the ageing of a culture.

Bridges & Darby (1933) showed that the *p*H of unbuffered *Drosophila* media decreases from about 5·8, where it lasts 1–2 days after preparation, to 3·6–3·8 during the subsequent 1–3 days, where it remains for about 3 days. Finally, it rises to 3·8–4·8. It was thought that these changes in *p*H might be responsible for the changes of recovery time correlated with the age of the culture. In experiments to test this the effect on recovery of slightly more extreme values of *p*H was investigated. Flies from the same culture were put simultaneously on a medium consisting of 10% molasses soaked in cellulose cotton (a material similar to that used by Spencer (1937)). The molasses were mixed with hydrochloric acid and calcium chloride powder or sodium hydroxide solution till a definite

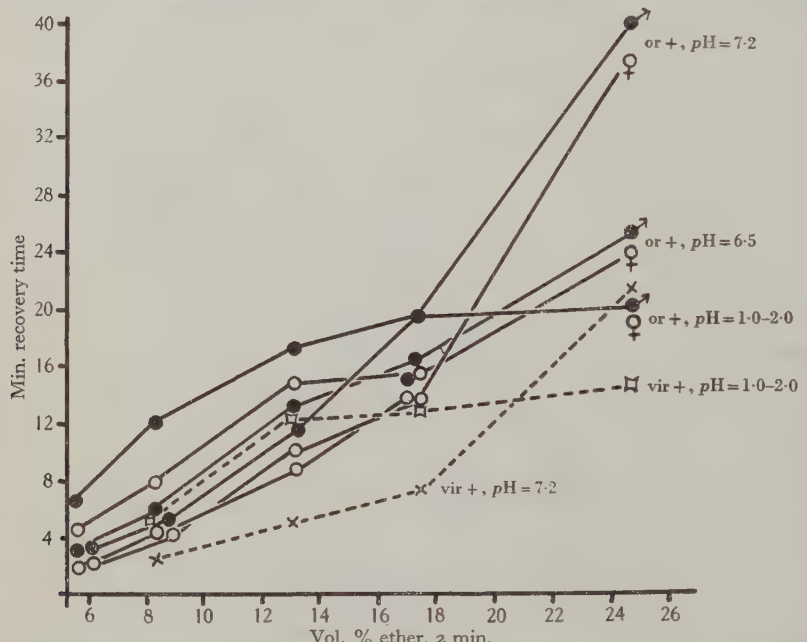

Fig. 8. Median recovery times of *D. melanogaster* and *D. virilis* kept on media with different *p*H.

*p*H was reached. This was measured by means of Clarke's colour indicators before and after the flies were put into it. The *p*H of the media changes very little during the presence of the flies (1–4 days).

Fig. 9 shows that the recovery time of flies of *D. melanogaster* and *virilis* is greatly influenced by the *p*H of their food.

After slight and medium etherization the flies kept on an acid medium recover much later than after being on a neutral or alkaline medium. After heavy dosages this behaviour is reversed. As the limiting concentration observed did not change very much, this behaviour will be regarded tentatively as a consequence of differences in the speed of ether permeation according to the *p*H. It is probable that the *p*H of the *Drosophila* tissues follows that of the food to some extent. According to this conception an acid state increases the influx of ether as well as the efflux, but as the latter lasts longer under the conditions of our experiment the recovery time is increased compared with the control

ıltiplication; during which time protein, or fat, metabolism increases and ·bohydrate metabolism becomes of less importance. In Tyrode solution, protein fat metabolism perhaps becomes more and more difficult, and the slowly grating cells are caused to live a much more hand-to-mouth existence upon ·arbohydrate diet extracted directly from the medium. From the point of view the relationship between aerobic glycolysis and growth, it is notable that lactic ·d production per unit of nucleoprotein is higher in Tyrode, where growth is nimal, than in embryo juice in which cell multiplication occurs freely. On the ıer hand, with these cultures the steady high lactic-glucose ratio in embryo juice ıy perhaps be significant. At present it is difficult to account for the great rise glucose consumption in Tyrode solution, which is not paralleled by a corre- onding rise in lactic acid production. Reference will be made later to this enomenon.

(2) Heart fibroblasts

Experiments upon exactly the same lines with fresh chick heart explants yielded sults which are in many ways different (Table 3). In the first place the initial ·cleoprotein phosphorus content of the cultures was much higher. In Tyrode lution the nucleoprotein progressively fell during the 4 days of culture, the fall

Table 3. *Data for heart 'fibroblasts'*

Medium and tissue	Time in hours	Mean N.P.P. in γ	Glucose utilized in γ	Lactic acid liberated in γ	Glucose / N.P.P. per hour	Lactic acid / N.P.P. per hour	Lactic acid / Glucose
·resh heart explants in Tyrode	0–20	1·68	270	110	8·0	3·3	0·41
	20–40	1·60	160	100	5·0	3·2	0·64
	40–60	1·50	150	80	5·0	2·7	0·54
	60–80	1·30	120	70	4·6	2·7	0·59
	80–100	1·12	100	60	4·5	2·7	0·60
·resh heart explants in embryo juice 15–40 %	0–20	1·94	320	160	8·3	4·1	0·49
	20–40	1·77	160	90	4·6	2·6	0·57
	40–60	1·70	160	80	4·7	2·8	0·60
	60–80	1·72	160	70	4·7	2·2	0·47
	80–100	1·92	160	70	4·2	1·9	0·45
th passage heart fibro-blasts in Tyrode	0–100	0·60	620	300	10·3	5·0	0·49
th passage heart fibro-blasts in 30 % embryo juice	0–100	0·73	660	370	9·1	5·1	0·56

ing more marked during the second 2 days (Fig. 7). Embryo juice made but little fference during the first 2 days, but determined a rise of nucleoprotein during e second 2 days, or at least there was no further fall. From the figures so far ailable it appears that the effect of the embryo juice was roughly proportional to e concentration of the juice, the values for 40 % being noticeably greater than ose for 20 % juice. It is noteworthy that fourth passage heart cultures (in a

shortening effect of hydrogen, nitrogen, carbon monoxide, coal gas and acetylene have been described by Kalmus (1935).

(*b*) *Shortening of the recovery time after hydrogen asphyxiation by application of carbon dioxide*

Drosophila flies immobilized by administration of hydrogen recover earlier when subjected to carbon dioxide. This effect resembles that of carbon dioxide in artificial respiration in man. It seems that immobilization through oxygen lack is only in small part due to the accumulation of carbon dioxide, other waste products (perhaps lactic acid) having a greater effect. The following results were obtained in an experiment designed to show this:

$T = 22°$ C. *D. subobscura pl pp op* flies immobilized in a stream of hydrogen for 2 min.: nine flies brought for 1 min. into 60% carbon dioxide (approx.) recovered in air after $2{\cdot}7 \pm 1{\cdot}03$ min. Nine control flies brought immediately into air recovered after $4{\cdot}7 \pm 1{\cdot}14$ min. The difference of 2·0 min. is significant. Similar results were recorded when the asphyxiation was caused by treatment in a vacuum.

(*c*) *Effects of carbon dioxide on recovery from hydrocyanic acid immobilization*

The stupefaction and recovery of *Drosophila* after treatment with hydrocyanic acid has been thoroughly investigated by Bliss & Broadbent (1935, 1936). Recovery time is a linear function of the sum of the logarithm of concentration and influx time. Table 3 shows that carbon dioxide treatment of *Drosophila* before exposure to hydrocyanic acid increases the recovery time, whereas carbon dioxide treatment of the hydrocyanic acid-immobilized flies shortens recovery.

Table 3. *Recovery times of* Drosophila melanogaster *after treatment with hydrocyanic acid* (15 *sec. in a killing bottle*) *and carbon dioxide* (90 *sec.*). $T = 21°$ C.

Treatment	CO_2 before HCN	HCN alone	CO_2 after HCN	CO_2 alone
Recovery of 5 out of 10 ♀♀	12 min. 16 sec.	9 min. 59 sec.	4 min. 37 sec.	2 min. 14 sec.

The first three recovery times were measured from the moment the flies were removed from hydrocyanic acid, the last one from the time they were removed from carbon dioxide.

(*d*) *Shortening of recovery from ether narcosis by application of carbon monoxide and coal gas before narcosis*

By subjecting *Drosophila* flies to carbon monoxide or coal gas a shortening of recovery time from a subsequent ether narcosis can be obtained. It seems that the carbon monoxide, by blocking some of the respiratory catalysts, reduces for some time the ability of the organism to make use of atmospheric oxygen and turns it over to a partly anoxidative metabolism. Haldane (1927) proved that a wax moth (*Galleria mellonella*) needs more oxygen for the maintenance of its motor activity in the presence of carbon monoxide than it needs in air. In *Drosophila* a slight sluggishness of movement can be observed for several hours after the flies have been exposed for 1 min. to coal gas. This is most striking at low temperatures where the chill coma temperature (Mellanby, 1939) appears considerably raised. It may be that the presence of the carbon monoxide renders the

flies more acid and therefore shortens recovery time. It is therefore plausible to assume that flies treated with carbon monoxide take on some of the qualities of newly hatched flies for several hours. The result of a typical experiment was as follows:

$T=22\cdot5^\circ$ C.: twelve 1–2 days old *D. subobscura* treated with carbon monoxide for 50 sec. and recovered after 3–5 min., were narcotized after 6 min. for $1\frac{1}{4}$ min. in an atmosphere of 10·86 vol. % ether. They recovered after $4\cdot25 \pm 1\cdot10$ min., untreated control flies after $5\cdot59 \pm 1\cdot12$ min., the difference being significant. Similar results were obtained with *D. melanogaster oregon* F_5, *D. simulans* and *D. subobscura*, but not with *D. virilis*.

(*e*) *The effect of previous narcotization on recovery time*

In certain circumstances, if the narcosis is not too heavy and the flies are allowed to recuperate completely on their usual food, no significant differences in the individual recovery times can be observed, as already shown on p. 241. Pearl and Parker (1922) showed that no sensible alteration of the duration of life follows the ether nárcosis, as practised by *Drosophila* workers, even when repeated four times. However, when heavy dosages of narcotic are applied recovery times are altered in the intervals before recuperation is complete. Two antagonistic effects can be observed which sometimes counterbalance each other.

The first is a *summation* effect; it occurs when the second narcosis starts before all the narcotic from the first has been completely eliminated from the animal's body, i.e. during the efflux remainder period. This is an effect easy to produce, and needs no further explanation. A second etherization towards the end of recovery time results in a marked excitation state followed by prolonged narcosis. Secondly, a *shortening* of recovery time occurs during the recuperation, presumably after all ether has gone, a result which is more difficult to demonstrate. However, as it is cumulative, the decrease in recovery time becomes significant after several repetitions of narcotization. The following figures denote recovery times of the same seven *D. melanogaster* flies etherized four times in 10·86 vol. % ether for $1\frac{1}{2}$ min.; the intervals between the narcotizations were about 20 min. in each case. The four recovery times were: $9\cdot12 \pm 1\cdot50$ min., $5\cdot93 \pm 1\cdot51$ min., $5\cdot01 \pm 1\cdot69$ min., and $4\cdot60 \pm 1\cdot23$ min. The recovery time of the eight control flies etherized for the first time and simultaneously with the seven flies in the fourth narcotization was $8\cdot18 \pm 1\cdot63$ min. The differences between the first and last recovery time (4·62 min.) and between the last recovery time of the seven flies and the recovery of the eight control flies (3·58 min.) are significant ($p < 0\cdot01$).

X. DIFFERENCES IN RECOVERY TIME OF SPECIES, RACES AND MUTANTS OF *DROSOPHILA*

There are definite differences in recovery from etherization in *Drosophila*, but although this fact is generally recognized, no one has so far attempted to analyse these differences. Most batches of flies taken from two bottles will differ significantly in recovery time, when carefully examined throughout the concentrations (see Fig. 5), owing to differences in age, *p*H of culture, proportion of sexes, etc. Neither are differences in the recovery of flies emerging from one culture always due to genetical factors. Thus the F_1 generation of *w* females and + males in *D. melanogaster* is composed of phenotypically white-eyed males and red-eyed females. As females recover earlier than males from low or medium ether dosages, the difference in recovery time between the red and white-eyed flies cannot

The glucose uptake by the tissue is also very different from that already described for osteoblasts. First, there is practically no difference between the uptake in Tyrode and that in embryo juice; in fact, when related to the nucleoprotein of the tissue the sugar uptake in the two media is identical. There is a preliminary rapid uptake, which is, however, not so rapid as in the case of osteoblasts, and then the uptake remains constant (Fig. 9) at about the same level as that reached by osteoblasts after 3 days in embryo juice. The lactic acid production follows a similar course and, as far as the figures show, the amounts are nearly identical in embryo juice and in Tyrode, and this seems to apply also to tissue which has been cultured for four passages in hanging-drop cultures, and therefore comparable in that way to the osteoblasts already described. There can be no doubt therefore that the carbohydrate metabolism of the two cell types is different in several ways. The first reaction to explantation is in both cases an increased glucose uptake, which in osteoblasts is greatly increased by embryo juice, but not in heart fibroblasts. Then osteoblasts in Tyrode require embryo juice to prevent the glucose utilization mounting to great heights, a phenomenon which is not shown by heart tissue. Finally, there is the curious loss of nucleoprotein phosphorus from heart fibroblasts in culture which can only be held in check by high concentrations of embryo juice.

The effects of epicutan on skin cultures

During the course of this work some experiments were being carried out upon the effects of epicutan upon the growth of skin. Epicutan is a substance extracted from embryonic tissues and on the market in the form of a powder (Fischer, 1939, 1940). A few experiments were therefore carried out on similar lines to those

Table 4. *Showing the glucose utilization, lactic acid production and nucleoprotein phosphorus content of chick skin tissue after 4 days of culture in a plasma coagulum bathed either with epicutan in Tyrode or in Tyrode alone. It is noticeable that carbohydrate metabolism is more intense in the Tyrode medium in which growth is less than in epicutan, but that the lactic-glucose ratio is higher in the growth-promoting medium, epicutan*

Medium	Tube	N.P.P. γ	Glucose mg.	Lactic acid mg.	Glucose (γ) / N.P.P.	Lactic (γ) / N.P.P.	Lactic / Glucose
Epicutan	1	3·08	0·704	0·225	228	73	0·32
	2	4·23	0·844	0·215	199	51	0·25
	3	3·10	0·685	0·215	220	69	0·31
	4	1·65	0·460	0·155	279	94	0·34
Tyrode	5	2·68	1·200	0·328	449	122	0·27
	6	1·34	0·825	0·155	615	115	0·19
	7	2·42	1·220	0·337	505	139	0·28

already described on osteoblasts and heart fibroblasts. Skin tissues were used from the embryo chick. Here again, in cultures in plasma and Tyrode solution alone, there was considerable glucose uptake which was much greater than that found in cultures with epicutan in the medium, both actually and in relation to the amount of nucleoprotein present. The lactic acid production followed a similar line, but

nce again the lactic-glucose ratio was higher in the growth stimulant (epicutan) ıan in Tyrode, though the ratio found was lower for skin than for osteoblasts nd heart. Epicutan caused some increase in nucleoprotein phosphorus, though ɔt as great as that produced in osteoblasts by 15 % embryo juice. Epicutan appears act upon the skin cultures in much the same way as a rather dilute embryo juice cts upon osteoblasts. The results are shown in Table 4.

DISCUSSION

It may now be appropriate to discuss the results reported in this paper with ference to data previously obtained and to data available from other sources, and attempt in a tentative manner an analysis of the position.

In the first place the significance of the nucleoprotein phosphorus measurements ıay be assessed. Such measurements as exist for the dry weights of cultures of steoblasts in 15 % embryo juice by Laser (1933 *a*) show a very good agreement with e present results. Using his figures for the increase in dry weight of cultures ıring the first 4 days, the relationship is set out in Table 5.

It is clear that in this case the nucleoprotein and the dry weight are closely lated, and the only figures available for the dry weights in Tyrode medium show ɔ increase over the first 4 days, which again agrees with the nucleoprotein hosphorus measurements (Laser, 1933 *b*). No similar figures are available for eart fibroblasts, where other factors may enter into the situation. It is possible ıat there may exist in the heart muscle cells some, perhaps extranuclear, nucleo-rotein which disappears during culture, and that when that has gone the cultures

Table 5

Age of culture (days)	Dry weight (Laser)	N.P.P.	Percentage increase of N.P.P. / Percentage increase of Wt.
0	65	0·4	
2	105	0·54	0·57
4	220	0·9	0·61

ould behave more like osteoblasts. It is notable that Berenblum *et al.* (1939), orking on tissue slices suspended in Tyrode solution, found that an initial decrease nucleoprotein phosphorus took place which they ascribed to autolysis. The sults recorded in the present paper show that the nucleoprotein content can only ith difficulty be maintained by high concentrations of embryo juice. It is well ıown that the mitotic rate of heart fibroblasts is lower than that of osteoblasts d high concentrations (40 %) of embryo juice are required to maintain a growth te equivalent to that produced by 15 % juice on osteoblasts. It would be interesting know if the dry weight of heart cultures follows the nucleoprotein content, cause, since the glucose consumption and the lactic acid production follow the cleoprotein phosphorus then, if the dry weight does not also follow it, the cleoprotein would acquire special significance in relation to carbohydrate etabolism. Further experiments are also necessary on fourth passage heart

3. The recovery time is increased by the time of influx and by the concentration of the narcotic. The shapes of the curves obtained in experimental series are logarithmic (carbon dioxide asphyxiation), straight line (ether in some species), concave, convex or sigmoid (ether). It is suggested that the different forms of ether recovery curves are different parts of essentially similar curves, which one might explain by the joint action of two macrophases, one aqueous and one lipoid.

4. Physiological factors determining recovery times are: (*a*) sex: females recover earlier from ether narcosis than males; (*b*) age: young flies recover earlier than older ones; (*c*) lack of food and moisture, which increases the recovery time; and (*d*) chemical reaction: flies kept on acid food remain longer narcotized than flies bred on an alkaline medium.

5. Carbon dioxide lengthens recovery from ether narcosis and hydrocyanic acid immobilization when applied before the influx time and shortens it when applied during recovery time. If administered during recovery, it also shortens the recovery from asphyxiation.

6. Carbon monoxide and coal gas administered before narcotization can shorten the recovery time from ether narcosis.

7. Under specified conditions corresponding to those used during narcosis by *Drosophila*-workers some differences in recovery time after etherization due to genetical differences could be established. Significant differences also exist between some *Drosophila* species, races and mutants in their resistance to carbon dioxide, asphyxiation and hydrocyanic acid gas.

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THE MECHANISM OF THE NERVOUS REGULATION OF THE CRAYFISH HEART

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(With One Plate and One Text-figure)

INTRODUCTION

The existence of nerves regulating the frequency of the heart beat in crustaceans has been repeatedly reported (Jolyet & Viallanes, 1892; Dogiel, 1894; Connant & Clarke, 1896; see also Dubuisson, 1934). Carlson (1905, 1906), studying the nerves of *Panulirus* more extensively, came to the conclusion that two nerve pairs from the suboesophageal ganglion innervate the heart, the anterior pair being inhibitors, the posterior accelerators. The situation in crabs seems to be very similar, but the crabs have two pairs of nerves, instead of one, with accelerating properties (Connant & Clarke, 1896). Another nerve to which a regulatory function has been ascribed is the nervus cordis, or nerve of Lemoine (Lemoine, 1868).

It was found that the preparation of the crayfish (*Cambarus clarkii*) heart as used in the investigation of the effect of ions (Cole, Helfer & Wiersma, 1939) was very suitable for an investigation of the function of the regulatory nerves. In this preparation the nerves can easily be prepared and stimulated under conditions which allow a simultaneous registration of the heart beat. In such preparations, it was found possible to locate, in addition, tracts in the oesophageal commissures which have an inhibitory and acceleratory function.

METHODS

To study the nervous regulation of the heart, the crayfish is first eviscerated by opening the carapace in front of the cervical groove, and most of the chitin from the dorsal and lateral sides in front of the groove is removed. The stomach and 'liver' are pulled out, care being taken that no digestive juice escapes into the body cavity. The green glands and the muscles of the mandibles are taken out and the cavity is washed with physiological solution (van Harreveld, 1936). In all cases the crayfish were first made clawless, since it was found that otherwise the claws invariably became entangled with the recording thread.

Three ways of mounting the preparation have generally been used.

In the first method the crayfish is clamped in a vertical position, tail down. In this position continuous perfusion with physiological solution is carried out, as described by Cole *et al.* In order not to submerge the nerves during stimulation, a hole can be made on the ventral side of the animal at the level of the heart. The heart beats were recorded with a light heart lever, connexion being made by attaching a small pinch clamp to the heart. In some preparations the nerves may be damaged by this attachment, and stimulation of the nerves becomes without effect.

which occur in this figure have been noted for fourth passage chick osteoblast cultures and for fresh heart explants during the first 4 days of culture in media of plasma and Tyrode and of plasma and embryo juice.

3. The figures for nucleoprotein content have been correlated with those for glucose consumption and lactic acid production and pronounced differences have been observed in the behaviour of the two types of tissue. The possible significance of the findings is discussed.

4. There is no absolute correlation between high growth rate and high sugar consumption or lactic acid production.

5. The data discussed are consistent with the idea that an increased protein metabolism, and a decreased carbohydrate metabolism result from the addition of embryo juice to osteoblast cultures. Embryo juice has no detectable effects on the carbohydrate metabolism of fresh heart cultures.

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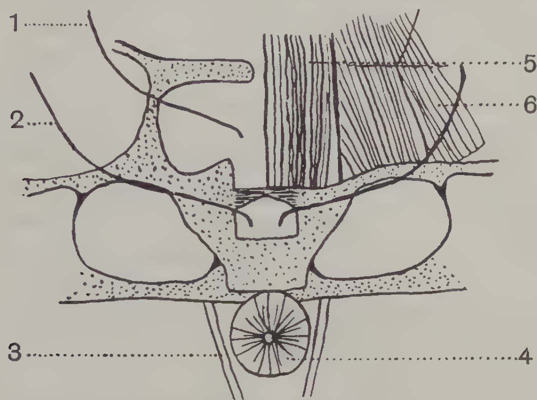
It is quite possible that this difference is not due to a different number of nerve fibres stimulated but to slight variations in the threshold of stimulation. Since preparation of single fibres could not be performed, it is not known how many inhibitory fibres are in each nerve, but the number is most likely very small and might well be only one.

Complete stoppage of the heart occurs only at rather high frequencies. In fresh preparations the necessary frequency is about 40 per sec.; in older ones it may rise to 90 per sec. Slowing of the frequency of the heart beat becomes evident only with frequencies



Text-fig. 1. Diagram showing the course of the inhibitor and accelerator nerves in the antero-ventral portion of the thoracic cavity showing: 1, accelerator nerve; 2, inhibitor nerve; 3, oesophageal commissures; 4, oesophagus; 5, flexor muscles; 6, extensor muscle.

above 10 per sec. This is partly due to the fact that at low frequencies the effect develops so gradually that it becomes difficult to distinguish it from other spontaneously occurring changes. Frequencies of about 20 per sec. result in a gradually established slower rhythm which may be constant for several minutes. With frequencies between this value and one which completely inhibits, an initial stoppage is obtained which remains longer the higher the frequency. After this stoppage is over a regular rhythm is usually estab-

Table 1. *Influence of the frequency of stimulation of the peripheral inhibitor. Stimulation of 15 sec. duration.*

Stimulation frequency per sec.	Heart rate per min.			Stimulation frequency per sec.	Heart rate per min.		
	Before	During	After		Before	During	After
60	112	0	112	15	112	108	112
45	112	72	112	10	112	112	111
30	112	81	113	20	111	96	110
20	112	104	112	60	108	0	105
45	112	76	113				

lished which is lower the higher the frequency of stimulation. Table 1 shows determinations for a certain preparation in which the frequency between tests was unusually constant over a long period. Pl. 1, fig. 1 shows a typical set of records obtained on stimulation with different frequencies.

Stimulation of either the left or right inhibitor results in identical effects; when both are stimulated simultaneously, a stronger inhibition results than on stimulation of either alone.

In a series of experiments action potentials were led off during peripheral inhibition. A hole was made in the carapace above the posterior part of the heart, and into it one lead-off electrode was inserted. The other was placed just anterior to the pericardial cavity. The action potentials were almost without exception single spiked, a '*T*' wave often following the spike. This '*T*' wave was, at least to a large extent, due to movement of the anterior electrode with each heart beat. During stoppage of the heart by inhibition, the electrocardiogram disappeared completely. When smaller beats occurred during inhibition, correspondingly smaller potential spikes were obtained.

The effect of stimulation of the peripheral accelerators

The accelerator nerves are more difficult to prepare than the inhibitors, since, on their entry into the main body cavity, they are covered by the flexor muscles in the thorax and emerge on the surface of the extensor muscles only after they have reached the more caudal part of the cavity (Text-fig. 1). These nerves would seem to correspond to the third superior nerves from the suboesophageal ganglion as described by Keim (1915) for *Astacus*. Near the heart they are in such close proximity to the inhibitors that selective stimulation is difficult. Preparations were made by cutting the insertion of the flexor muscles and carefully bending these back. Most of the experiments were performed by gently pressing the electrodes against the exposed nerve; care was taken to stimulate with rather weak currents to prevent simultaneous stimulation of the inhibitors. In a number of experiments the latter were removed for a considerable length in order to diminish further this possibility. Experiments in which the exposed part of the nerve was stimulated by lifting it on micromanipulated electrodes showed the same results.

During stimulation of the accelerator, both the frequency and amplitude of the beat are increased. In some cases during maximum stimulation the amplitude of the beats is lower than normal. In other hearts acceleration gave rise to prolonged beats, and the picture resembled more or less that of an incomplete tetanus. It is never possible to accelerate a heart above a certain limit; this maximum response tends to remain constant over short intervals, so that minor differences in frequency before stimulation have little effect on the frequency during stimulation. If a heart attains a slower rhythm after a longer period, acceleration cannot induce it to reach its former maximum, nor can a heart which is beating relatively slowly be speeded up to the same frequency which a heart, beating normally at a higher rate, can reach.

As with inhibition, the accelerating effect diminishes during prolonged stimulation. After a shorter stimulation the frequency may stay higher than normal for some time after the stimulation, returning gradually to its former value (Table 2). There is no difference in the effects of stimulation of the right and left accelerators. Simultaneous faradic stimulation of both has very little stronger effect that that of one; in almost every instance the frequency was only a few beats per minute faster than on stimulation of either one alone.

A study of the influence of the frequency of stimulation reveals that the accelerators differ markedly from the inhibitors, since low frequencies have a noticeable effect on the accelerators but not on the inhibitors. Stimulation rates as low as 1 per sec. gave an increase in frequency (Table 2A), and even a single stimulus could sometimes shorten the pause between two beats to a measurable degree. In such sensitive preparations frequencies of above 30 per sec. did not result in a further pronounced increase. In older

or less sensitive preparations, higher frequencies are necessary to obtain the same effects. Table 2B shows a typical set of responses of a heart to different frequencies of stimulation, frequent returns to 200 stimuli per sec. being made to test the maximum response. Pl. 1, fig. 2 shows a series of records in which the effects of different frequencies of stimulation are illustrated.

The action potentials led off during acceleration are, of course, more frequent than normal. In addition, there is a definite increase in the size of the spike, which accompanies the increase in the mechanical beat size. In the cases where prolonged beats occurred during acceleration, it was found that the accompanying action potentials showed more tops, and the more pronounced the mechanical prolongation was, the clearer the tops could be distinguished.

Table 2. *Influence of the frequency of stimulation of the peripheral accelerator. Stimulation of 15 sec. duration.*

Stimulation frequency per sec.	Heart rate per min.			Stimulation frequency per sec.	Heart rate per min.		
	Before	During	After		Before	During	After
A 7	52	66	58	B 30	48	64	52
5	50	64	56	200	56	66	58
2	50	62	54	20	52	62	58
1	54	64	62	200	52	72	56
B 200	48	68	48	20	52	60	52
100	40	64	48	7	52	56	52
60	40	64	48	7	44	48	48
45	32	68	44	200	44	72	66
200	40	68	44	7	48	56	52

Simultaneous stimulation of the inhibitor and the accelerator

In a number of experiments, the effects of simultaneous stimulation of the accelerator and inhibitor nerves were investigated. The combined effects were roughly the average of the individual effects. For instance, one preparation with a normal rate of 18 per 15 sec. was accelerated to 21 by maximal stimulation of the accelerator nerve and was depressed to 12 by stimulation of the inhibitor with a stimulation frequency of 30 per sec.; when both were stimulated at the same time, the beat rate was 16. Another preparation treated similarly had a normal beat rate of 15, an accelerated rate of 19, an inhibited rate of 9, and with both nerves stimulated together, a beat rate of 14 per 15 sec. When inhibition is complete, however, simultaneous stimulation of the accelerator is generally without immediate effect, although cases have been found in which a few escape beats were induced.

The after-effects of inhibition

After a heart has been stopped by maximal stimulation of the inhibitor, it generally returns gradually to its former frequency. This phenomenon is more apparent in hearts, which are normally beating slowly. Immediately after stopping stimulation of the inhibitor, the heart invariably beats once. However, the pause between this beat and the following one is usually longer than normal, and for the first few beats this interval between beats may even lengthen somewhat before gradually returning to normal (Pl. 1, 1:60).

When acceleration and inhibition are applied and released simultaneously, the after-effect of inhibition largely disappears, although acceleration may not manifest itself at the time of stimulation of the nerves (Pl. 1, fig. 3). The delayed action of the accelerator nerve under these conditions suggests liberation of a substance, which can exert its influence at a later time.

The effects of eserin and acetylcholine

Perfusion of the heart with eserin (10^{-5}) yielded no obvious effect; in a few cases the beat frequency was slightly raised, in others it was lowered. Comparison of the acceleration produced by maximal stimulation of the accelerator nerve before and during perfusion with eserin shows that eserin enhances the effect of acceleration (Table 3). Eserin has no influence on the effectiveness of inhibition.

Table 3. *Comparison of the effects of stimulation of the accelerator nerve of eight different hearts before and after eserinization. Stimulation of* 120 *per sec. for* 15 *sec.*

	Before eserinization. Heart rate per min.			After eserinization. Heart rate per min.		
	Before	During	After	Before	During	After
A	24	104	92	28	116	84
B	84	96	88	72	108	84
C	92	104	96	92	108	84
D	36	84	48	32	104	68
E	68	80	68	64	88	72
F	72	84	72	32	92	60
G	72	92	76	68	116	80
H	72	84	76	72	104	80

In confirmation of Welsh (1939*a*) and Davenport (1941) perfusion with acetylcholine gave an immediate increase in frequency and amplitude of the beat. During perfusion complete inhibition can be obtained, but somewhat higher frequencies than those effective before the addition of acetylcholine are necessary. A certain preparation, for instance, could be completely inhibited by a stimulus of 40 per sec., but a stimulus of 50 per sec. was necessary during acetylcholine perfusion. Upon release of inhibition, the heart may enter a partial tetanus of brief duration.

The maximum degree of acceleration obtained by perfusion with acetylcholine and by stimulation of the accelerator is approximately the same. In Pl. 1, fig. 4, the slightly greater number of beats obtained by acetylcholine stimulation as compared with nervous stimulation is represented by a larger number of small alternate beats.

The nerve of Lemoine

This nerve is part of the so-called sympathetic system, which connects with the central nervous system mainly at the commissural ganglia situated about half-way between the 'brain' ganglion and the suboesophageal ganglion. There has been considerable discussion whether the nerve makes connexion with the heart or whether it stops at the anterior arterial valve (Lemoine, 1868; Police, 1908; Keim, 1915; Alexandrowicz, 1932; Heath, 1941). A regulatory influence has been ascribed to this nerve (Young, 1878; Plateau, 1880; Moquart, 1883) and also has been denied it (Jolyet & Viallanes, 1892; Connant & Clarke, 1896; Carlson, 1905). The negative evidence of the latter workers was obtained by stimulation of the 'brain' after transection of the oesophageal commissures.

In order to investigate the effect of the nerve of Lemoine on the heart, another method of preparation has been used. The tail was removed from the animal and the posterior side of the heart exposed, leaving the 'liver' completely intact. The carapace was carefully removed for a small distance on top of the stomach, exposing the nerve which is very thin, and which runs in the median line. The preparation was mounted head downwards and the clamp fastened to the posterior side of the heart. No effect whatsoever was noticed in such preparations upon stimulation of the exposed nerve with various frequencies. As a control the peripheral inhibitors and accelerators were subsequently stimulated in these preparations, with the expected effects. Several variations were made in order to exclude possible damage of the nerve by preparation, but in no case was any change in heart-beat size or rate found.

The inhibitors in the central nervous system

Stimulation of the oesophageal commissures frequently results in an immediate stopping of the heart. By dividing the commissure into bundles and stimulating these separately, it was found that this inhibitory function was the exclusive property of a very thin fibre bundle, consisting of three fibres at most. It is likely that only one of these, which is thicker than the others (diameter about 30 μ), is solely responsible. Single fibres in the commissure are, however, much sooner damaged than those in peripheral nerves, and this conclusion is, therefore, not absolutely certain.

The experiments on the effect of this inhibitory tract have been varied in many ways. In a number of experiments the peripheral accelerators were cut, and the central inhibitors more or less prepared. There was no difference between the left and the right side. On subsequent cutting of the peripheral inhibitor on one side, this situation was still unaltered, but the effect was diminished. Cutting of the other peripheral inhibitor abolished all inhibitory effect of central stimulation on the heart. It is thus certain that each of the inhibitory tracts in the centre makes connexion with both the left and right peripheral inhibitors. Stimulation of the central stump of a cut peripheral inhibitor never results in inhibition through the other inhibitor, which proves that synapses intervene.

In another set of experiments the influence of the frequency of central stimulation was investigated. It was found that the effects are much less constant than with stimulation of the peripheral inhibitor. One difference which was regularly observed was that central stimulation had in many cases a pronounced after-effect, when such an effect was absent or slight in peripheral stimulation. This indicates an after-discharge of the inhibitory ganglion cell. In fresh preparations the use of very low frequencies will often give slowing or complete stoppage; in older ones there is usually not much difference between the result of using the same frequencies on central or peripheral application. After cutting one of the peripheral inhibitors, it is necessary to use a considerably higher frequency to obtain the same effect as before the cutting; the required frequency may be about twice as high. Simultaneous stimulation of both central inhibitors results in a stronger effect than that obtained by stimulation of only one.

In these experiments proof is given that there is a tract from the 'brain' to the suboesophageal ganglion, which gives inhibition exclusively. It is certain that this is not the only tract in the central nervous system which connects with the peripheral inhibitors. In preparations in which the superior ganglion no longer functions, stimulation of the

tail still results in heart inhibition, showing the presence of a presumably similar tract in the lower part of the central nervous system. This inhibition also disappears on cutting the two peripheral inhibitory nerves.

The accelerators in the central nervous system

Preparation of the central accelerators is less satisfactory than that of the inhibitory tract, mainly because of the much shorter survival time. After the peripheral inhibitors have been cut, stimulation of the unprepared oesophageal commissures results in acceleration. After cutting the peripheral accelerators this effect disappears, although in some preparations a very prolonged stimulation has ultimately resulted in some speeding up of the heart. It is clear, however, that this is a different effect, which may be due to the release in the blood stream of a substance liberated somewhere else in the body.

The two accelerators are most likely the only nerves with this function in the crayfish. As in the case of the inhibitors, both oesophageal commissures contain fibres which are connected with each of the two accelerators, and no difference was found between the two sides. As mentioned, preparation of bundles in the commissures is difficult because of the short survival time, therefore no definite statements are possible about the specificity of the tracts, but it was conclusively shown that only a small part of the oesophageal commissure possesses this property.

In a number of experiments it was investigated whether or not cutting the peripheral inhibitors or accelerators results in a persistent change in the frequency. These experiments were largely negative: in the prepared animal there is thus no evidence of an inhibitor or accelerator tone.

Stimulation of a whole oesophageal commissure with different frequencies in preparations with the four peripheral heart regulators intact showed that low frequencies of stimulation cause a short lasting inhibition which is gradually displaced by an accelerated rhythm. This is always found with frequencies lower than 20 per sec. High stimulation frequencies (e.g. 60 per sec.) result in a stoppage of the heart for some seconds, after which a gradual increase in the beat rate takes place, but in these cases the frequency remains lower than the original.

DISCUSSION

From the results obtained by a number of workers with the hearts of both *Limulus* and the decapod crustaceans, and from the results described in this paper for the crayfish heart, a hypothesis with regard to the mechanism of the crayfish heart beat is proposed. This hypothesis is meant to serve only as a means of combining as many observations as possible. Most of its parts have already been proposed by others in the same or similar form.

The crustacean heart beat is of neurogenic origin. The intrinsic ganglion cell pool has a spontaneous rhythm. For each heart beat a series of impulses (volley) is sent down the nerve fibres connecting the ganglion cells with the heart muscle fibres. The frequency of the heart beat is determined by the frequency of volleys. The frequency of volleys is altered by the influence of the regulatory nerves by means of neurohumours. The acceleratory neurohumour is acetylcholine; the inhibitory neurohumour is unknown. During acceleration, the number of impulses in each volley as well as the frequency of volleys increases; during inhibition the reverse is true.

The neurogenic origin of the beat in *Limulus* has been generally accepted since the classic experiments of Carlson (1905–6), notwithstanding a few experiments which tend to show that a myogenic rhythm may be present under certain conditions (e.g. Hoshino, 1925). The similar experiments of Alexandrowicz (1932) have indicated the neurogenic basis for the beat of the crustacean heart. The unquestionable presence of ganglion cells adds weight to this view. In general, the results described by us and other workers conforms much better to the theory of neurogenic origin than that of myogenic.[1]

The spontaneous rhythm of the ganglion cell pool is not as well established. That such an automatic rhythm may be present in structures comparable to these has been shown by Weiss (1941). Gerard & Libet (1939) also are of the opinion that synchronizations like these are frequent in central nervous systems and bring evidence that the factor responsible for the synchronization is electrical rather than chemical.

That volleys of impulses are set up in the nerve fibres leading from the ganglia to the muscle fibres of the heart has been shown in *Limulus* by Heinbecker (1933), and by Armstrong, Maxfield, Prosser & Schoepfle (1939). In the case of the crustacean heart, the evidence is only indirect (see, however, Rijlant, 1932). The partial suppression of the beat height during slowing by inhibition and the increase in height during acceleration can be readily explained by a decrease and increase, respectively, in the frequency of nerve-fibre discharges in each volley, accompanied by a change in the total number of impulses per volley. The prolonged beats sometimes found in older heart preparations can be explained by an excessively prolonged duration of each volley. In maximum acceleration, the rest periods between different volleys may become so small that the heart fibres never completely relax, resulting in tetanic contractions.

It seems likely that acetylcholine is the medium by which the accelerator nerves produce their effect because of the identity of the effects of nerve stimulation and perfusion with acetylcholine. This view is held by a number of authors (Welsh, 1939*a*, 1939*b*, in *Carcinus* and *Panulirus*; Davenport, Loomis & Opler, 1940, in *Astacus*; Davenport, 1941, in *Cancer*; Obreshkove, 1942, in *Daphnia*). Further support of this view is given by the fact that eserin enhances the effect of stimulation of the accelerator nerve. It seems clear that the acceleratory effect is mediated through the ganglia rather than through the muscle fibre directly. Garrey (1942) has shown that in *Limulus* acetylcholine does not act on heart-muscle fibres, although it accelerates the heart if the ganglion is perfused; likewise, acetylcholine has no effect upon crustacean peripheral muscle fibres (du Buy, 1935; Bacq & Nachmansohn, 1937).

The humoral nature of the inhibiting substance is more doubtful. None of the substances tried so far have given a comparable result. Its humoral nature can be inferred only from the after-effect of inhibition, an effect which is often but by no means always present. It is not inconceivable that inhibition has a different, e.g. electrical, nature, but this will have to be studied further. No evidence has been found that the inhibition of the heart is like the typical peripheral inhibition of the crustaceans. The close correlation between the action potentials and the beat size does not fit in with peripheral inhibition. The location of the inhibitory effect is, without much doubt, in the ganglion.

Many authors have claimed that the electrocardiogram of the crayfish regularly shows

* In this connexion it may be pointed out that in the peripheral muscles of the crustaceans, conduction in the muscle fibres may well be absent (see Wiersma, 1941). If this is true, too, for heart-muscle fibres, the neurogenic origin would be imperative.

a number of tops and have concluded that the heart beat is tetanic in nature. Dubuisson (1934), however, has shown that under normal conditions the electrocardiogram exhibits a single top. Our observations wholeheartedly agree with those of Dubuisson. It has been shown that in peripheral nerve-muscle preparations in crustaceans a quick succession of impulses gives rise to a smooth, high action potential rather than to a series of tops (Wiersma & van Harreveld, 1938). The same might be the case here. Variation in the number and the frequency of the impulses in the intrinsic nerve fibres would then readily explain the respective increase or decrease in size of potentials during acceleration and inhibition. The many topped action potentials of the prolonged beats would reflect the irregularities in the discharge of the ganglion cells.

SUMMARY

A preparation is described in which the peripheral inhibitor and accelerator nerves of the crayfish (*Cambarus clarkii*) heart can be separately or simultaneously stimulated. The effect of different frequencies of stimulation were investigated; complete stoppage of the heart was obtained only with rather high frequencies (45 per sec.); maximal acceleration, with lower frequencies (30 per sec.). No difference in effects between left and right nerves was found. Perfusion with acetylcholine and stimulation of the accelerator nerve produce identical effects. Perfusion with eserin does not influence the normal heart beat but enhances the effect of acceleration.

The nerve of Lemoine has no regulatory influence on the heart.

Special inhibitory and acceleratory tracts have been prepared in the suboesophageal commissures. Each of these tracts makes heterolateral as well as homolateral connexions. The influence of the frequency of stimulation on the central tracts has been studied.

An hypothesis with regard to the mechanism of the crayfish heart beat and its control is presented.

The authors wish to express their gratitude to Mrs Mary Lissner Stuppy for her invaluable assistance during the early stages of this work.

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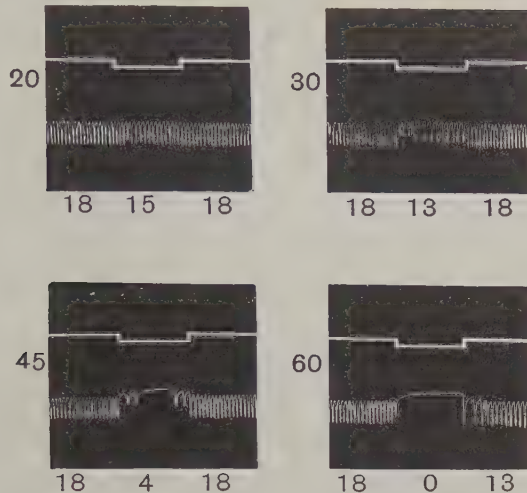

Fig. 1

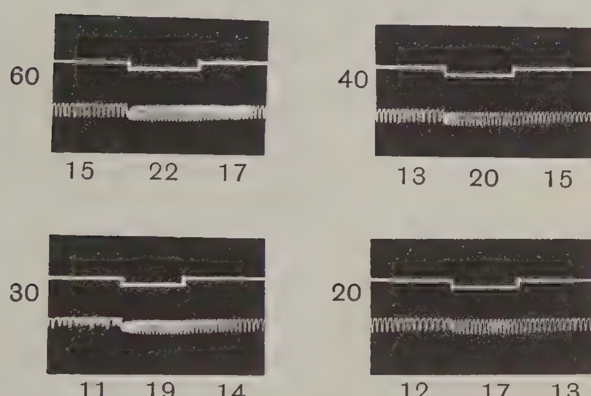

Fig. 2

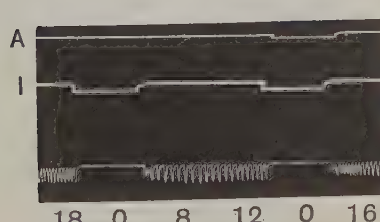

Fig. 3

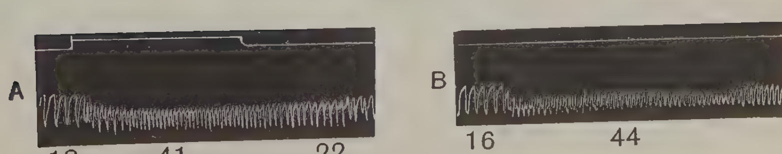

Fig. 4

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EXPLANATION OF PLATE 1

Fig. 1. Influence of the frequency of stimulation of the inhibitor nerve on the heart rate and beat size. The number to the left of the stimulation signal is the stimulation frequency per second. In this and all subsequent figures the numbers below the records show the number of beats per 15 sec., and the duration of stimulation is 15 sec., unless otherwise indicated. In this and all the subsequent figures, the contraction is recorded as a downward stroke.

Fig. 2. Influence of the frequency of stimulation of the accelerator nerve. Symbols same as in Fig. 1.

Fig. 3. After-effect of inhibition alone compared to that after simultaneous stimulation of the accelerator nerve. Stimulation signals: *I*, inhibitor (60 per sec.); *A*, accelerator (60 per sec.).

Fig. 4. Comparison of the effect of maximal stimulation (120 per sec. for 30 sec.) of the accelerator nerve (*A*) with perfusion with a few drops of 10^{-6} acetylcholine (*B*). The beat rates are given for 30 sec. intervals.

THE OUTWANDERING OF CELLS IN TISSUE CULTURES OF NERVES UNDERGOING WALLERIAN DEGENERATION

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(With One Text-figure)

INTRODUCTION

When a nerve is sectioned the whole peripheral stump undergoes Wallerian degeneration, and a small part of the central stump near the cut also shows degenerative changes. While axons and myelin degenerate during this process the Schwann (or sheath) cells proliferate and in the peripheral stump become arranged in the well-known 'bands of Büngner'. The endoneurial fibroblasts also proliferate. A study of the outwandering from explants of degenerating nerves in tissue culture should throw light on the changes in Schwann cells and fibroblasts, the physiology of which is little understood. It may also be of practical importance in nerve surgery, for it seems that outward migration of the Schwann cells from the cut surfaces of the nerve, which will be reflected by their outwandering in vitro, plays an important part in ensuring adequate healing (Young, 1942).

Several workers have grown Schwann cells in vitro, notably Ingebrigtsen (1916), Chlopin (1939) and Murray, Stout & Bradley (1940). Of these only Ingebrigtsen was concerned with the changes in the cells during Wallerian degeneration. He studied the peripheral stump during the first 19 days of degeneration, using as a criterion of activity the percentage of explants which showed any outwandering. Our work may be considered as an expansion of Ingebrigtsen's suggestive beginning. We have investigated regions of both stumps, degenerated for periods of up to a year, and we have used for estimating activity a method which is more easily interpreted than that of Ingebrigtsen. The present paper is a general survey of quantitative differences of cell outwandering in vitro between various regions of the severed nerve and between nerves at different times after they have been severed. We are much indebted to Mr J. Z. Young for suggesting that we should undertake this investigation, and for criticizing the manuscript.

TECHNIQUE

The explants were taken from the sciatic nerve of adult rabbits. In most of the experiments the peroneal branch was used; in a few, the tibial branch. There was no significant difference of behaviour of the two branches. At an initial operation a piece of varying

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length was cut from the nerve with sharp scissors in the thigh region at about the level of the third trochanter of the femur. After a varying interval of time we made a second operation and removed lengths of the central and peripheral stumps of the nerve for culture. In each experiment we cultured a number of explants from each of several different parts of the stumps of the severed nerve, as listed below.

In most experiments our only provision to prevent reinnervation of the peripheral stump was to make the gap between stumps large; but when nerves were left to degenerate a long time the possibility that reinnervation had occurred was investigated by treating histological sections by Bodian's method for staining of axons. In none of the peripheral stumps used for this paper were more than a very few isolated axons found.

In some experiments the central stump was killed at the initial operation to ensure that it did not reinnervate the peripheral stump. This was done by injecting the central stump with a 1 % aqueous solution of crystal violet (a method of axon destruction used by Guttmann & Medawar, 1942). The method was not entirely suitable for our purpose, because we found that the proximal part of the peripheral stump was sometimes a little damaged.

The ordinary hanging-drop method of tissue culture was used. Two explants were placed on each cover-slip. The medium consisted of fowl plasma and chick embryo extract. Through most of the series of experiments we made the extract from a dried embryo brei (see Peacock & Shukoff, 1940) kindly prepared for us by Dr E. Chain of the Pathology Department, Oxford University. We used this at a concentration representing 20 % of pure embryo juice, the diluent being Pannet and Compton's saline. The dried preparation was reasonably constant in action during the time it was in use. It was already several months old when we made most of our experiments, and had lost some of its original power. In a confirmatory set of experiments we used fresh embryo extract, which was considerably more stimulating. The epineurium was removed from the nerve before culture. The nerve was then divided into explants about 1 mm. long, consisting of the whole or half of the cross-section. Cultures were kept at 38° C., and in most experiments were cultured 4 days, in some cases 3–7 days, before fixing. Conditions of culture were kept as constant as possible throughout the series of experiments, and the different times of degeneration we used were well randomized with respect to dates at which we made the experiments. In many experiments normal and degenerated nerve, or nerves of different periods of degeneration, were cultured simultaneously.

Cultures were fixed in 4 % formaldehyde and stained in Ehrlich's haematoxylin. Wandering cells, fibroblasts and Schwann cells occurred in the zone of outwandering. The wandering cells were sporadic in occurrence, and we do not report on them here. Fibroblasts were sometimes very numerous, and the amount of their outwandering was estimated by eye, using an arbitrary scale with 4 divisions, the index 4 representing the highest number. The amount of Schwann cell outwandering was obtained by counting the Schwann nuclei in the zone of outwandering. This could be fairly accurately done because the number of nuclei in most cultures was small and it seldom exceeded 1000. Since mitosis of Schwann cells in the zone of outwandering is very rare the nuclear count corresponds closely to the number of nuclei which have wandered out. The length in profile of the transverse cut surface of the explant was measured, and the Schwann cell activity of a given region of nerve was then expressed as the mean number of Schwann nuclei which have wandered out per mm. of this measurement (usually during 4 days

in vitro). Such a standardized measure of activity, even though imperfect, is an advantage, since there was considerable variation of the size of the nerves, but it was not worth applying to the rough estimates of fibroblast activity. It may be added that the nuclear count reflects approximately the distance the Schwann cells have migrated into the clot.

Note on identification of Schwann cells

The Schwann cell in vitro has been described by Ingebrigtsen (1916), Chlopin (1939) and Murray *et al.* (1940). It is unnecessary to add anything to these descriptions. At a later date we intend to publish a detailed account of the cytology of the Schwann cell in vitro. It is not difficult to distinguish the majority of Schwann cells from fibroblasts; but difficulties sometimes arise when separating very elongated fibroblasts, such as may occur in the substance of the clot, from the more richly cytoplasmic Schwann cells. A series of intermediate forms can be traced. So the decision is sometimes arbitrary; but these doubtful cells are very few in number compared with the clearly identifiable cells. Since it may have introduced a systematic error into our results, a more serious difficulty is that a new type of cell appears in the cultures from long-degenerated stumps. Although typical Schwann cells and typical fibroblasts still occur, many of the cells are of extraordinarily ramifying and straggling form. They have characteristics of, and grade into, both typical Schwann cells and typical fibroblasts. We believe them to be Schwann cells and have counted them as such; but if we are mistaken, our counts of Schwann cells from 139 days onward are over-estimated by about 50 %.

THE ACTIVITY OF NORMAL NERVE

In the conditions of culture used in these experiments, normal (i.e. not predegenerated) nerve is very inactive. Nineteen experiments with normal nerve were made, and of these thirteen showed no outwandering whatever. Of the 213 explants cultured in these experiments, 191 (90 %) were blank after 4–6 days in vitro; the remaining twenty-two explants, which were confined to six of the experiments, realized between them a total of thirty Schwann nuclei and a few fibroblasts. Half of this total came from explants taken at different times from the right and left nerves of one particular rabbit. The mean number of Schwann cells per mm. of explant was about 0·1 and of fibroblasts about 1, for all normal nerve cultures.

One of the active nerves was sectioned and examined histologically. A group of degenerated fibres was found in it. The activity shown by some normal nerves can perhaps be accounted for by the occasional occurrence of a few degenerated fibres.

In view of the sporadic occurrence of activity in normal nerve, we made, when necessary, cultures of normal nerve from the same rabbit simultaneously with those from previously cut nerves. These controls were always blank. For purposes of comparison with previously cut nerves, normal nerve can therefore be considered inactive.

THE PERIPHERAL STUMP

Several different regions of the peripheral stump were cultured, the nerve having always been cut about half-way down the thigh. The regions used were as follows:

Bulb, the terminal swelling derived from the hernia produced by the retraction of the perineurium and epineurium from the nerve fibres at the cut surface.

Traumatic, a region 5 mm. long immediately peripheral to the bulb.

Standard, the region stretching from the traumatic region to the biceps blood vessels.

Knee, the peroneal nerve at knee level.

Shank, anterior tibial branch of peroneal in shank.

Schwannoma region, the connective tissue immediately central to the bulb, into which cells migrate from the bulb forming eventually a tumour-like mass.

SCHWANN CELL ACTIVITY IN PERIPHERAL STUMP

(*a*) *Traumatic and standard regions*

Most of our data are derived from the traumatic and standard regions, and in the early stages of the work these two regions were not distinguished. So we shall first consider the traumatic and standard regions jointly.

Table 1. *Outwandering of Schwann cells* (*nuclei per mm. explant*) *and of fibroblasts* (*expressed as units on an arbitrary scale*) *from explants of nerves at various times after cutting, cultured 4 days in vitro.*

Days after cutting	Rabbit	No. of explants	Mean no. of Schwann nuclei per mm. explant	Mean Fibroblast index
1	X	16	0	0
2	Y	16	0·2	0·05
3	X	24	0·9	0·17
4	Y	14	6·4	1·1
5	—	16	0·9	0
6	—	4	51	0·1
9	—	2	65	1·2
*10	Z	12	71	0·7
11	—	4	53	2·3
13	—	8	158	1·8
16	G	6	198	2·9
19	—	7	350	0·5
*20	Z	8	462	2·4
*20	O	8	392	2·3
23	—	7	247	3
25	G	6	255	3·6
*25	O	12	470	2·7
29	—	6	125	1·6
*35	O	14	188	0·6
39	K	16	111	1·6
60	K	20	94	1·1
*72	—	14	159	1·6
*97	O	12	130	0·4
*139	—	22	116	0·6
344	—	16	50	0·3
402	—	25	35	0·23

* Cultures made in specially active medium.

In column 2 the designations of rabbits used in 'paired' experiments are given; in these cases, right and left peroneal nerves were cultured simultaneously and in the case of rabbit O peroneals (25 and 97 days) and tibials (20 and 35 days) were all four cultured simultaneously.

Changes of Schwann cell activity with time of degeneration shown by these regions of the nerve are given in Table 1; they are typical for the rest of the peripheral stump. Only explants cultured 4 days in vitro are included. The results of eighteen other experiments, in which explants were cultured for a longer or shorter time, substantiate these changes. Column 4 gives the mean number of Schwann nuclei outwandered per mm. of explant and these values are plotted against time of degeneration in Fig. 1. The experiments indicated by * in Table 1 were made more recently and all within a short time

of each other (four were made simultaneously on rabbit O). They differ from previous experiments in that freshly prepared embryo extract was used. The extract proved to be more stimulating than the dried form, so the results cannot be combined with the previous ones. They are shown in a separate curve in Fig. 1.

Fig. 1 is of course constructed with data from many different nerves, but it may be considered to show approximately changes of activity with time in a single nerve after section. After an initial period of inactivity or of slight activity (the time of onset of activity is analysed below), a rapid rise starts at about 4 days of degeneration and carries

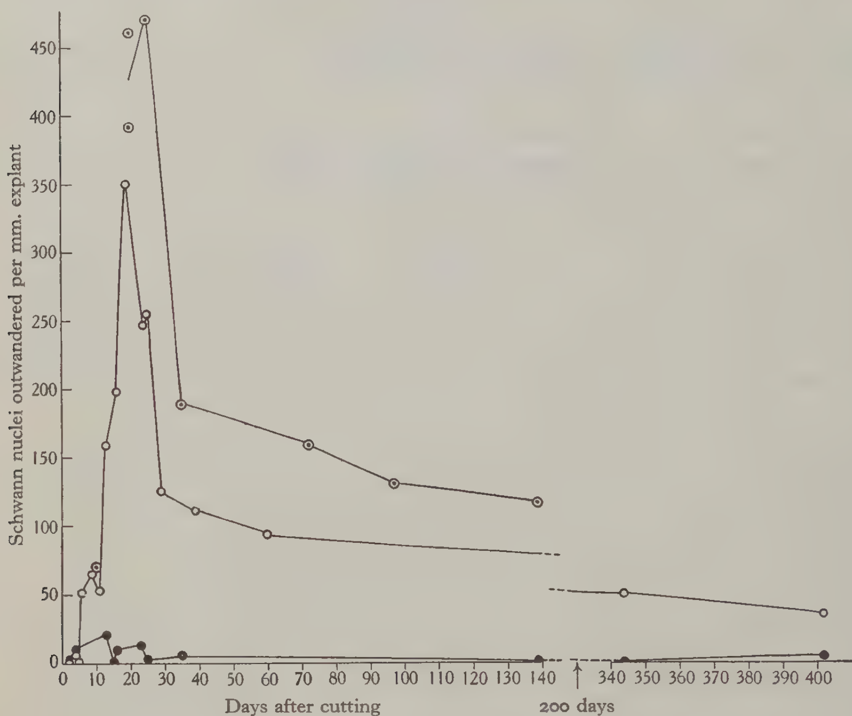

Fig. 1. Outwandering of Schwann cells from explants of nerves at various times after cutting. ● central stump; ○ peripheral stump; ⊙ peripheral stump cultured in highly active medium.

the activity to a peak at about 19–25 days. At the peak, activity is about 40 times as great as at 4 days. After the peak there is a decline, probably at first nearly as steep as the rise, but soon becoming less steep; after about 60 days the fall is very slow. After 1 year of degeneration activity is still considerable, being 5–7 times as high as at 4 days.

Since it is clear that there is considerable variation of experimental conditions and from animal to animal, useful confirmation of the general trend of the curve is provided by those experiments in which two nerves from the same animal but of different degeneration times were cultured simultaneously, and therefore in conditions as nearly identical as possible. The results of the 'paired' experiments (indicated in Table 1, column 2, by the rabbit's protocol designation) were as follows (the figures refer to days after cutting; the sign indicates which time had the greater outwandering): 1 < 3; 2 < 4; 10 < 20;

16 < 25; 39 > 60. In one case (rabbit O) peroneals and tibials were simultaneously cultured; of the peroneals, 25 > 97, of the tibials, 20 > 35; while comparing a tibial with a peroneal, 20 < 25 and 35 > 97. The difference in activity between the members of a pair was in three instances statistically significant; between 10 and 20 days of degeneration (mean activity and standard error 71 ± 20 and 462 ± 34 respectively); between 20 and 35 days of degeneration (392 ± 67 and 188 ± 26 respectively); and between 25 and 97 days of degeneration (470 ± 57 and 130 ± 12 respectively).

Comparison of traumatic and standard regions. It is known that the region of the peripheral stump immediately adjacent to the cut undergoes precocious degeneration (Cajal, 1928). The effect of this on Schwann cell activity was investigated in twenty-two experiments comprising 130 traumatic and 166 standard explants.

In each experiment explants from the two regions of a single nerve were cultured simultaneously, and their activity compared. We found that the mean activity of the traumatic region explants was greater than that of the standard region explants in seventeen of the experiments (owing to the small numbers of explants counted, however, the difference was only rarely statistically significant in an individual experiment). In four other experiments the mean activities were the same. In the remaining experiment, at 1 day of degeneration, we found no activity of either region. In the early stages of degeneration (2–4 days), the mean activity of the traumatic region explants is very much higher (4–20 times) than that of the corresponding standard region. With further degeneration it is a small but rather constant amount higher, averaging 154 ± 16 % of that of the standard region, a significant difference. It is clear that the raised activity of the traumatic region persists for 2–3 months after the initial cut; and possibly, though our data are insufficient, for a year.

We found indications that a traumatic effect was also produced by recutting a nerve which had already degenerated. Two experiments of this kind were made. A nerve of 15 days' degeneration was recut and cultured after a further 18 days, when the new traumatic region was 510 % of the standard region; similarly a nerve recut after 30 days' regeneration and cultured after a further 18 days had a new traumatic region 145 % of its standard region.

Onset of activity. The fact that the activity of the traumatic region is so markedly in advance of that of the standard region during the early stages of degeneration calls for more detailed investigation of the time of onset of activity in the two regions after the initial cut.

After 4 days in vitro both standard and traumatic regions of one day of degeneration were entirely inactive. At 2 days of degeneration the standard region was inactive, but the traumatic region had produced an average of 0·5 Schwann nucleus per explant. At 3 days of degeneration the standard region produced an average of 0·2 Schwann nucleus per explant, the traumatic 3·5. Thereafter the activity of both increased, the traumatic region maintaining its lead, but proportionately a much reduced one.

A longer cultivation of the apparently inactive nerves was undertaken. Even after 8 days in vitro both regions of the stump at 1 day of degeneration are quite inactive. But while no activity was detectable in the 2-day degenerated standard region after 4 days in vitro, slight activity was shown after 7 days in vitro (mostly in the form of filaments of Schwann cell cytoplasm, without nuclei). Strictly parallel experiments done with normal undegenerated nerve showed complete inactivity after 7 days in vitro. It can

therefore be inferred that the traumatic region becomes active at a slightly earlier time of degeneration than the standard region (probably late on the first day), and that the standard region first acquires activity on the second day of degeneration.

(*b*) *Bulb*

The terminal *bulb* of the peripheral stump was not used to derive the curve of Schwann cell activity shown in Fig. 1 owing to its variability and the frequency with which the Schwann cells are obscured by fibroblasts. In spite of considerable variability the changes of the Schwann cell activity of the bulb with time of degeneration repeat in general form the course of activity shown in Fig. 1; activity starts on the second day, and from the fourth day rises fast to a peak at 15–25 days after cutting, thereafter declining. During these changes, but excluding the very long degeneration times of 344 and 402 days, the bulb is approximately as active as the traumatic region (though it varies between 50 and 400% of this region); it is therefore (in twelve out of fourteen comparable experiments) more active than the standard region. However, in the two experiments with nerves of very long degeneration (344 and 402 days) the activity of the bulb was about one-fifth or less of that of the rest of the peripheral stump. The bulb was studied in 25 experiments, comprising 90 explants.

(*c*) *Knee and shank*

The standard, traumatic and bulb regions discussed above are all parts of the sciatic nerve in the thigh. It seemed possible that activity might differ in the more peripheral parts of the nerve. In several experiments in which the nerve was cut in the usual place, pieces were taken for culture from the peroneal nerve at knee level (16 experiments, 100 explants) and from the anterior tibial branch of the peroneal half-way down the shank (9 experiments, 52 explants).

In both regions the changes of activity with time of degeneration are approximately the same as those of the thigh region, though a good deal more irregular. There is the same rise to a peak, at 20–23 days' degeneration, and the same fall after the peak, at first rapid, then slow. The general level of activity proved to be higher than that of the standard region of the same nerve in nine out of eleven experiments on the knee region and in all eight experiments on the shank where comparison is possible. When the activities of the knee and shank regions were expressed as percentages of those of the standard region, much the highest values were obtained in the experiments at the shortest degeneration times. These were for the knee region 640% at 5 days and 500% at 7 days: for the shank 500% at 5 days and 760% at 10 days. At longer degeneration times the percentages decreased. They varied for the knee region (13–402 days) between 30 and 200%, the mean and its standard error being $120 \pm 19\%$; and for the shank region (21–97 days) between 112 and 260%, the mean and its standard error being $210 \pm 26\%$. In comparable experiments the activities of the shank and knee regions were not significantly different from each other.

It should be noted that the shank region, separated by about 8 cm. from the cut in the nerve, cannot be affected by the initial operation except in so far as Wallerian degeneration is concerned.

(*d*) *Schwannoma region*

It is known that Schwann cells migrate out from the cut surface of the peripheral stump (i.e. the bulb) in vivo, and infiltrate the neighbouring connective tissue, forming

a tumour-like mass (Nageotte, 1913) in the later stages of degeneration. We cultured the tissue from immediately beyond the tip of the bulb after various times of degeneration to test the activity of these emigrant Schwann cells. Twenty-two such experiments comprising 105 explants were made.

Explants from the schwannoma region are of variable activity, because it is not homogeneous nervous tissue, and some of the explants turn out to be connective tissue only. For this reason detailed quantitative treatment is not useful. But the general trend of activity with time of degeneration is clear. No Schwann cells appeared from explants taken from nerves of 6 days' degeneration or less (five experiments). At 7 and 11 days of degeneration only very few Schwann cells were observed (two experiments). But from 13 to 25 days of degeneration (seven experiments) there were many explants which produced abundant Schwann cells, fully as many as the bulb or traumatic region. With longer degeneration (60–139 days, six experiments) activity is less, far below that of the peripheral stump proper, e.g. at 139 days the schwannoma region had an activity 20% of that of the bulb. Finally, in the experiments at 344 and 402 days of degeneration, when a massive tumour-like schwannoma had formed, the explants were almost completely inactive. At 344 days the mean number of Schwann nuclei (twelve explants) was 1% of that of the peripheral stump proper; at 402 days no Schwann cells outwandered (five explants). In these experiments the bulb had also only very slight activity.

It is notable that when activity is high Schwann cells often grow from the schwannoma explants in the form of isolated massive trunks, which dissociate into separate cells as outwandering proceeds. This is presumably because the schwannoma contains thick cords of Schwann cells.

Latency and rate of outwandering

As we might expect, the latency (i.e. the period between the setting up of the cultures and the first appearance of outwandering) varies inversely with the amount of activity of the explant. (Schwann cells wander out before or at the same time as fibroblasts in all regions except the bulb and schwannoma.) Changes of latency are not, however, the only cause of changes of activity with time of degeneration. There are also changes of the *rate* of outwandering when the period of latency is over. The outwandering per mm. of explant *per day of the period of outwandering* changes in a way generally similar to the activity for 4 days in vitro (Table 1, column 4) so far considered. In particular, there is a fall of rate similar to the fall of activity after a peak at 19–25 days of degeneration. In fact, latency is negatively correlated with rate of outwandering and changes in both together lead to the observed changes of amount of activity

But the relationship between latency and rate of outwandering is not constant at all times of degeneration. We have compared explants of the same rate of outwandering but from nerves of different degeneration times. (Explants with a period of outwandering of 2 or 3 days' duration were used; they had been in vitro 3–6 days, according to their period of latency.) We found that, in the later (post-peak) stages of degeneration, latency is, for the same rate of outwandering, greater than in early degeneration. For a rate of between 20 and 60 Schwann nuclei per mm. per day, nerves of 7–16 days of degeneration have a mean latency of $1{\cdot}3 \pm 0{\cdot}13$ days (nineteen explants) and of 33–402 days of degeneration a mean latency of $1{\cdot}7 \pm 0{\cdot}07$ days (fifty-seven explants), the difference being

significant. Similarly, for a rate of outwandering of between 60 and 90 Schwann nuclei per mm. per day, nerves of 23–30 days of degeneration have a mean latency of $1 \cdot 1 \pm 0 \cdot 04$ (thirteen explants) and of 33–139 days' degeneration a mean latency of $1 \cdot 7 \pm 0 \cdot 14$ (fifteen explants), the difference being significant. Thus both the pre-peak and the peak explants have a shorter latency for the same rate of outwandering than the post-peak explants. Our data do not conclusively show that there is a progressive increase of latency during the post-peak decline of activity. With a rate of outwandering of 20–60 Schwann nuclei per mm. per day, explants from nerves of 33–60 days of degeneration have a mean latency of $1 \cdot 6 \pm 0 \cdot 10$ (twenty-nine explants) and those from nerves of 139–402 days of degeneration a mean latency of $1 \cdot 8 \pm 0 \cdot 074$ (twenty-eight explants). The difference is hardly significant ($P = 0 \cdot 05$).

FIBROBLAST ACTIVITY IN THE PERIPHERAL STUMP

The fibroblasts are often too numerous to count, and the fact that they are intermixed with Schwann cells makes estimation by area of outwandering inaccurate. Only an approximate analysis of their outwandering can therefore be made, using an arbitrary scale of 4 indices, the index 4 representing the highest number of cells. The fibroblast activity of a nerve is expressed as the mean fibroblast index per explant.

The changes in the mean index of fibroblast activity with time of degeneration are shown in Table 1, column 5. Column 4 shows the Schwann cell activity of the same explants. The general trends of the Schwann cell and fibroblast figures are closely similar, though the fibroblast activity is a good deal more variable both between explants and between experiments. At different degeneration times the proportion of fibroblasts to Schwann cells shows no significant changes. With respect to the time of degeneration at which activity begins there is no discernible difference between fibroblasts and Schwann cells.

When traumatic and standard regions are considered separately we find that the changes of their fibroblast activity follow substantially those shown for the two regions jointly in Table 1. The difference between fibroblast activities of the traumatic and standard regions parallels the difference between their Schwann cell activities. At 2–4 days of degeneration the fibroblast activity of the traumatic region is considerably higher (7–50 times) than that of the normal region, while at later stages it is only about twice as high.

In the *bulb* the activity of fibroblasts does not follow that of the Schwann cells. There is a rapid rise of fibroblast activity in the early stages of degeneration. In the experiments at 4 days of degeneration fibroblast activity was already half the maximum reached. Thereafter there is a slow rise accompanying the rapid rise in Schwann cell activity. In the post-peak period of Schwann cell activity there is no sharp fall in fibroblast activity which is well maintained for at least 4 months of degeneration. Cultures after 344 and 402 days of degeneration were, however, practically inactive in fibroblasts, as they were in Schwann cells. In five experiments for which satisfactory comparable data are available, the bulb is twice as active in fibroblasts as the traumatic region, and 4 times as active as the standard region. Thus the ratio of fibroblasts to Schwann cells is about $1\frac{1}{2}$–2 times as big as in the traumatic or normal regions.

The fibroblasts of the *knee* region are like those of the standard and traumatic regions in that they follow the Schwann cells in their changes of activity with time of degenera-

tion; but they differ in that (in eight out of ten experiments) they form a relatively higher proportion of the outwandering—roughly $1\frac{1}{2}$ times as many fibroblasts per Schwann cell as in the traumatic and standard regions. The absolute level of fibroblast activity is about twice that of the standard region.

Data for the *shank* region are not sufficient to show anything but that the usual curve of activity with time of degeneration is followed, and that the proportion of fibroblasts to Schwann cells and the absolute level of fibroblast activity are probably higher than in the standard region.

Fibroblast outwandering in the *schwannoma* is variable like Schwann cell activity. Its changes with time of degeneration are rather similar to those of the bulb. At 4, 5, and 6 days after cutting a few explants already showed considerable fibroblast (and no Schwann cell) activity. Activity probably increased further up to about 20 days. The subsequent decline was much more marked than in the bulb, and activity was poor in experiments at 2 months after cutting. Almost complete inactivity was reached at 344 and 402 days after cutting. The activity we obtained from explants of the peripheral bulb suggests that many of the fibroblasts of the schwannoma will have wandered there from the cut surface of the stump.

THE CENTRAL STUMP

The following regions of the central stump were cultured:

Bulb, which has the same position and origin as the bulb of the peripheral stump.

Distal region, extending for 3–4 mm. above the bulb.

Proximal region, central to the distal region, separated from it by $\frac{1}{2}$–2 cm.

Neuroma region, the connective tissue immediately distal to the bulb, into which cells and axons migrate from the bulb, forming eventually a tumour-like mass, corresponding to the schwannoma of the peripheral stump.

SCHWANN CELL ACTIVITY IN THE CENTRAL STUMP

In the early stages after cutting, activity of the Schwann cells in the central stump is confined to the region immediately near the cut end. This part, consisting of the *bulb* and *distal regions*, is so small that the data have been grouped for comparison with the peripheral stump. The changes of activity with time after cutting are shown in Table 2 and Fig. 1. The explants which supplied these data were all 4 days in vitro with the exception of those at 344 and 402 days of degeneration, which were longer (their values at 4 days were approximately half those given).

Changes of activity with the course of degeneration are not nearly so marked as in the peripheral stump. Activity is first found at 2 days of degeneration. After 4 days of degeneration it has already reached about half its maximum. There does not appear to be a sharply defined peak of activity as in the peripheral stump, but rather a plateau of raised activity. A series of 'paired' experiments in which nerves of the same rabbit, cut at different times previously, were simultaneously cultured, together with some other cultures kept longer than 4 days in vitro and therefore not included in Table 2 and Fig. 1, suggest that the highest part of the plateau may be early, between 5 and 10 days after cutting. The results of the 'paired' experiments were as follows (the figures refer to days after cutting, the sign indicates which time had the greater outwandering);

1 < 3, 2 < 4, 7 > 13, 16 > 25, 23 > 400, and 3 > 344. Of the seven experiments in which the explants were kept in vitro for more than 4 days the two highest mean activities occurred at 6 and 7 days after cutting. But although the position of the highest point in the activity curve is uncertain (and it is quite possible that different nerves may differ widely in this), it is clear that the maximum activity of the central stump is less than 10% of the maximum activity reached by the peripheral stump. The data indicate that with increasing time after cutting there sooner or later sets in a very slow decline in activity. A year after cutting, activity is still present to the extent of about 10% of the maximum.

When the activity of the central stump is compared with the activity of the corresponding region, cultured at the same time, of the peripheral stump (i.e. the peripheral bulb and the traumatic regions) of the same nerve a clear change with time after cutting is apparent. At 2 days after cutting in one experiment the central stump mean was

Table 2. *Outwandering of Schwann cells from explants of terminal 5 mm. of central stumps at various times after cutting of nerve*

Days after cutting	No. of explants	Mean no. of Schwann nuclei per mm. explant	Days after cutting	No. of explants	Mean no. of Schwann nuclei per mm. explant
1	8	0	16	11	10·0
2	8	3·5	23	8	13·0
3	8	0·1	25	12	3·4
4	8	10·5	35	8	6·1
5	6	2·8	139	12	1·2
13	12	20·4	344	12	0·5
15	12	1·9	402	12	4·5

900% of the peripheral stump mean, in a second experiment 250%. At 3 days after cutting it was 143% in one experiment, 5% in another. At 4 days after cutting it was 186% in one, 95% in another. At 5 days after cutting it was 23%. Thereafter it never exceeded 12% of the peripheral stump. Activity is therefore much greater in the central stump than in the peripheral stump in the early stages after cutting. We have not shown that it starts earlier. Likewise the peripheral bulb and traumatic regions are much more active in this stage of degeneration than the standard region; the central stump is therefore far more active than the standard region of the peripheral stump. But when the peripheral stump starts its rapid rise at 4 days of degeneration, it quickly overtakes the central stump, and is thereafter more active: at its peak at 19–25 days, it is 20–100 times more active than the central stump; and after a year, about 10 times.

The central bulb and the stump immediately above it separately show the same behaviour as that described for them jointly. They differ in that the bulb usually has the higher activity. In thirteen out of nineteen experiments the bulb is ahead, averaging in all about twice the activity of the stump proper.

We found that the proximal region, more central than the distal region so far discussed, was inactive in the early stages after cutting but that later it acquires some activity. This region was cultured in 18 experiments comprising 125 explants. Of nine experiments, covering 1–19 days after cutting, only one showed a trace of activity: this consisted in the appearance of a few cytoplasmic processes. Of nine experiments, covering 23–402 days after cutting, only one shows no activity, and this was cultured

23 days after cutting. All experiments made after 23 days show activity in this region of the central stump. Normal (undegenerated) nerves were cultured simultaneously with four of the eight positive cultures. All normal explants were entirely inactive.

The activity of this proximal region of the stump is very small, however, and has a very long latent period. Adequate comparison with the more distal part of the central stump is not usually possible, because the latter was fixed after about 4 days in vitro, and at this time the former was still blank. In three experiments, at 23, 344 and 402 days of degeneration, in which all regions of the central stump were fixed at the same time, the proximal central stump averaged 35% of the distal.

The neuroma region was cultured in nine experiments comprising sixty-four explants. In experiments at 15, 23, 23, 35, 53 and 139 days after cutting, the neuroma region showed always a moderate degree of Schwann cell activity, sometimes more, sometimes less than the central bulb. Activity is far less than that of the schwannoma region; actual figures are available only for one experiment, at 139 days, and in this there were 10 times as many Schwann cells in the schwannoma region. The other experiments showed a similar order of difference. Three massive neuromata, taken from nerves cut 298, 344 and 402 days previously, were quite inactive, showing that there is a decline in activity with long degeneration, as in the schwannoma.

FIBROBLAST ACTIVITY IN THE CENTRAL STUMP

In the central *bulb* the activity of fibroblasts at different times after cutting of the nerve is very similar to that of the peripheral bulb. The highest activity we found was at 4 days after cutting, and up to 2 months after cutting little decline was apparent. In central bulbs cultured a long time after cutting (139, 344 and 402 days) however no fibroblasts outwandered. In the central bulb there is, compared with the peripheral bulb, higher fibroblast activity, just as there is higher Schwann cell activity, between the second and fifth days after cutting. But after 5 days of degeneration the central bulb has, in nine out of ten experiments, a slightly lower fibroblast activity, averaging 80% of that of the peripheral bulb. Later than 5 days after cutting, the ratio of fibroblasts to Schwann cells is much higher in the central than in the peripheral bulb, for the fibroblasts in the central bulb are only slightly less active than those in the peripheral bulb, while the Schwann cells are far less active. Up to 5 days after cutting the ratio is about the same in the two bulbs.

The active terminal part of the central stump (*distal region*) just above the bulb shows exactly the same trends as the central bulb. Fibroblast activity is very low or absent beyond 3 months after cutting. When compared with the corresponding region of the peripheral stump (traumatic region) its activity is found to be equal, or slightly higher in the first few days after cutting, but subsequently always lower, averaging 30% of that of the peripheral traumatic region.

The slight Schwann cell activity which appears late after cutting in the proximal part of the central stump is not associated with any fibroblast activity.

Our data on fibroblast activity of the *neuroma* are too incomplete for comparative purposes, but a decline to zero is fairly well established about a year after the cut. Between 15 and 53 days after cutting there were always considerable numbers of fibroblasts in the outwanderings (five experiments), at 139 days there were very few, and at 298, 344 and 402 days there were no fibroblasts, as there were no Schwann cells.

CONCLUSIONS AND DISCUSSION

The nature of activation

We have found that when explants from the stumps of a severed nerve are cultured in vitro, the number of Schwann cells and fibroblasts which wander out varies according to the region of the stumps from which the explants are taken and the length of time which has elapsed since the original section was made. Normal (i.e. not previously severed) nerve is usually completely inactive; so, we found, was nerve taken 1 day after it had been cut, whatever the region cultured. On the second day after section a very slight amount of activity is apparent in both peripheral and central stumps, and up to the fourth day corresponding regions of the two stumps show a similar, usually small, increase in activity. With longer periods between section of the nerve and culture of the stumps further changes of activity occur, but differently in the two stumps. In the peripheral stump (none or very little reinnervation having occurred) all regions tested, including parts 8 cm. away from the original cut, show a very rapid rise in Schwann cell and fibroblast activity, beginning at the 4th–5th day (except for the bulb fibroblasts, which show an earlier rise) and reaching a high peak at about the 20th–25th day after section. This initial rise of activity is in agreement with the results of Ingebrigtsen (1916) who, investigating the percentage of explants showing any activity, found that this index increased from 17% on the 5th day after section to 82% on the 19th day. He found no outwandering before the 5th day. Ingebrigtsen probably used a medium of far less growth-promoting power than ours. He did not experiment on nerves which had undergone longer degeneration, nor on central stumps. In peripheral stumps taken more than 25 days since the nerve was cut we found a fall in activity with time since section, rapid at first but soon becoming slower. At 2 months after cutting the level of activity is back to that of the 10th–12th day after cutting (one-third of the peak activity) while during the next 10 months it falls another 50%, to the same activity as that of roughly the 7th day after cutting.

In the central stump on the contrary, activity is at first confined to the few mm. at the cut end, and shows no rapid changes with time since cutting. After an initial rise a maximum is reached probably between the 5th and 10th days, but at a level which, except in the case of bulb fibroblasts, is negligible compared with that of the peripheral stump (equivalent perhaps to the peripheral stump at the 5th day); activity then slowly falls, but is still not zero after one year. After 23 days since section and up to the longest time tested (402 days) we found that the central stump 1–2 cm. proximal to the cut, previously inactive, developed a very slight activity.

The spatial distribution of the activity corresponds to that of the degeneration of the nerve fibres, which as is well known starts in the whole length of the peripheral stump and in the tip (1–2 cm.) of the central stump soon after the nerve is severed (see Cajal, 1928). It must be assumed that activity is the direct result of this degeneration. The occurrence of a very small amount of activity occasionally in undegenerated nerve and consistently in the more central parts of the central stump during the later stages after cutting, may be independent of degeneration. But we believe that the occasional activity of normal (undegenerated) nerve is no more than can be accounted for by the occurrence of a few degenerate fibres (Duncan, 1930) and in one instance we verified the presence

of degenerate fibres in an active normal nerve. As for the proximal part of the central stump, it is said (see Spielmeyer, 1929) that when no reinnervation occurs a slow degeneration or atrophy of individual fibres sets in, which would perhaps account for the slight activity which we found to occur after the 23rd day after cutting. Degeneration of Wallerian type is probably rare here, the changes being rather a diminution of fibre size, including the elimination of part of the myelin.

It is notable that in the peripheral stump (except in the bulb) where the changes of activity are pronounced, both Schwann cells and fibroblasts change in substantially the same way with time of degeneration (see Table 1). Both are presumably affected, directly or indirectly by the same changing stimulus. It is a reasonable hypothesis that this stimulus is a chemical one which is present during the early stages of Wallerian degeneration, contemporaneously in fact with the destruction of the major part of the nerve fibre. This occurs between the 4th and 20th days of degeneration, when the rise of activity is maximal. The stimulus presumably ceases to be present from about the 25th day onwards, and the result is a sharp fall in activity, not to zero, but, for reasons as yet unclear, to a moderate and slowly decreasing level. The persistence of this low level of activity is peculiar to the atmosphere of the nerve stump. The cells which outwander from the cut end of the peripheral stump in vivo, and eventually form the schwannoma, show the same rise to a peak of activity during the early stages of degeneration; but the subsequent fall is more rapid and activity is zero after a year of degeneration. The almost complete absence of outwandering in these schwannomata of long standing is doubtless connected with the fact that they are extremely fibrous, with few cells, as sections of the explanted material showed. Holmes & Young (1942) suggest that in such schwannomata the Schwann cells which originally formed part of them may have atrophied.

Since the terminal 1–2 mm. of the central stump undergoes a degeneration which is quite similar to the Wallerian degeneration of the peripheral stump, it might be expected to show the same changes of activity with time since cutting as the peripheral stump. That it does not do so can probably be ascribed to the presence of growing axons, which is the most important difference between central and peripheral stumps. But although the growing axons perhaps prevent the activity in the central stump from reaching a high level, they do not reduce it to zero even a year after cutting. The persistence of activity in the central stump recalls that in the peripheral stump, although it is at a much lower level; and as in the schwannoma, the cells which have wandered out from the cut end of the stump to form the neuroma do not maintain their activity after long degeneration.

The $\frac{1}{2}$ cm. of the peripheral stump immediately adjacent to the cut has a higher activity than the next $\frac{1}{2}$ cm. during at least the first 97 days of degeneration. It is known that degenerative changes begin earlier in the first few mm. of the peripheral stump, and in the corresponding region of the central stump, than in the rest of the peripheral stump. Correspondingly, activity is precociously high in these regions (particularly so, for an unknown reason, in the central stump); and, as a result, during the rise in activity in the peripheral stump, the first $\frac{1}{2}$ cm. is more active than the second. The fact, however, that activity remains higher in the first $\frac{1}{2}$ cm. at and after the peak of activity, instead of undergoing a precocious decline, must mean that the stimulus given to the cells in this region is not only precocious but also greater. This is also indicated by the fact that stimulation was obtained in the neighbourhood of the wound when an already

degenerated nerve was recut (though we have only done two experiments to test this). There appears to be therefore a distinct and additional traumatic stimulus, similar perhaps to that which activates fibroblasts when connective tissue is wounded, which is superimposed on the Wallerian activation near the cut end of the peripheral stump. It must be assumed, since the trauma affects the ends of the two stumps equally, that the condition is the same in the central stump.

The traumatic activation affects fibroblast and Schwann cells equally in the peripheral stump just below the terminal bulb. But in the bulb itself the number of fibroblasts is increased proportionately to the number of Schwann cells, especially in the early stages of degeneration (2nd–5th days). This is also true of the central bulb, where the number of fibroblasts is almost the same as in the peripheral bulb, although the number of Schwann cells (after the first 3 days of degeneration) is far smaller. The simplest explanation is that there is an early invasion of fibroblasts, stimulated by the wound, from the perineurium or other adjacent connective tissue, through the cut surface of the stumps into the bulbs. Later the direction of the migration is of course reversed, and cells leave the bulbs to form the neuroma and schwannoma. The schwannoma, like the bulb, shows a high fibroblast activity in the first few (4–6) days of degeneration, with no Schwann cell activity until after the 6th day. Whether these are the fibroblasts which invade the bulb is unknown.

We found that after degeneration of a year, the peripheral bulb had lost far more of its activity than had the rest of the peripheral stump. This is no doubt connected with the extensive fibrosis of the Schwann bundles which occurs in late degeneration close to the lesion (see Holmes & Young, 1942). The fibrosis in its turn may be related to the intense fibroblast activity characteristic of the bulb region in the earlier stages of degeneration. The loss of activity in the schwannoma is perhaps to be similarly explained.

Significance of activity

Clearly the changes in activity which the Schwann cells and fibroblasts show in vitro with increasing time of degeneration is a result of changes in the physiology of the nerve, which result from degeneration. We do not yet know anything further about these physiological changes, and it is not possible to correlate them clearly with the known changes of these cells during degeneration in vivo. Some correlation of mitotic activity and outwandering would be expected, since they have important features in common (pseudopodial activity, response to same stimuli in tissue culture). The literature suggests that the temporal correspondence in the changes of the two activities is not close, mitosis starting in the Schwann cells and probably in the endoneurial fibroblasts at the 4th day of degeneration, reaching its maximum at about the 6th–9th days and ceasing after the 15th–20th day (Cajal, 1928). Roughly speaking, however, the rise to the peak of outwandering activity coincides with the total duration of mitotic activity. It is probable that some migration of the Schwann cells takes place in the degenerating nerve in vivo, for instance during the formation of the Büngner bands (Holmes & Young, 1942).

However, it is highly probable that the changes in activity found in vitro will be directly reflected in one aspect of the behaviour of the cells in vivo: the outwandering from the cut surface of the nerve stumps into the surrounding connective tissue. The changes with time of this outwandering in vivo during the building up of the neuroma

and schwannoma cannot be directly inferred from our experiments. But if a degenerated peripheral stump is recut, the new in vivo outwandering from the cut surface will, it is highly probable, vary according to the curve of activity with degeneration time which we have obtained in vitro. The actual amount of outwandering will not necessarily correspond, owing to the difference of medium in which the cells grow; and the proportionate changes may not be exactly the same, owing to possible differential effects of the media; but it is unlikely that the general trend will differ significantly. Holmes and Young (1942), by measuring the length of the schwannoma produced from a cut nerve in vivo, after various degeneration times, have in fact obtained direct evidence on this point which is in general concordance with our conclusions.

Does the activity shown during the time spent in vitro represent the physiological state of the nerve at the moment of explanation: or do the processes of Wallerian degeneration proceed in vitro as they would in vivo, correspondingly increasing the activity of the cells? It is clear that if activation does proceed in vitro, it does not do so at the in vivo rate. Normal, i.e. undegenerated nerve (with a few exceptions) and nerve on the first day of degeneration, do not develop activity even though kept in vitro quite long enough (8 days and more) to do so if they changed at the rate they do in vivo. Deterioration of the medium is not the cause of this, since subculturing during this period also failed to elicit any outwandering. An experiment in which explants of an early stage of degeneration were subcultured after 4 days in vitro, and then grown another 4 days, showed that there was a slight rise in activity during the second period, but only a fraction of what would have occurred in vivo. We conclude therefore that, at least in the early stages of degeneration, activity in vitro represents fairly closely the physiological state of the nerve at the time of explanation.

Practical bearing

It has been pointed out that Schwann cell activity in vitro may be expected to reflect the Schwann cell outwandering from the cut end of the nerve in vivo. In the repair of nerves by suture or graft a successful junction is probably formed by a vigorous outwandering of Schwann cells from the peripheral stump or from the graft (Young, 1942; Holmes & Young, 1942). We find that, except for a short initial period, the longer a nerve is left before surgical repair is undertaken, the less active the Schwann cells of the peripheral stump are likely to be in forming a junction. In the rabbit the optimum time for repair from the point of view of Schwann cell outwandering is not later than 25 days after the initial lesion. Further, our results suggest that immediate suture would not be as favourable as suture delayed for a few (say 10–20) days, in order to allow the development of a fairly high Schwann cell activity at the time of suture. In this way there would be less likelihood that the Schwann cell junction will be hindered by the prior development in the suture-line of serious fibrosis. Such fibrosis is a likely consequence of the numerous active fibroblasts which we found in the bulb and nearby connective tissue from 4 days after the nerve was cut. The same argument indicates the use of predegenerated grafts, provided predegeneration is short (10–20 days).

These conclusions are in entire agreement with those of Holmes & Young (1942), who found from experimental suture that long delay after the initial lesion before surgical interference is inimical to good repair, but that immediate suture is not so effective as somewhat delayed suture. Further, Sanders & Young (1942), comparing autografts of

fresh nerve with autografts predegenerated 6–9, 14–16 or 25–28 days, found a smaller delay of the growing axons at the junction of graft and central stump with predegenerated than with fresh grafts; a difference which they regard as suggestive though not statistically significant.

It may prove possible to apply our results with reasonable assurance to the human without experimental analysis by a study of the histological correlations of the activity curve we have obtained in the rabbit.

SUMMARY

1. The technique of tissue culture has been applied to a study of the physiological changes undergone by the cells of a severed nerve. The sciatic nerve of adult rabbits was cut in the middle of the thigh and pieces of the central and peripheral stump were explanted at varying times after the original cut. The 'activity' of a part of a nerve is expressed as the amount of outwandering of the Schwann cells and fibroblasts after 4 days in vitro.

2. In general, except in the terminal bulbs (derived from the herniated ends of the cut nerve) Schwann cell and fibroblast activity changes in a similar way.

3. In the conditions of our experiments normal (undegenerated) nerve shows activity only very rarely. Such activity as does sometimes occur can be explained by the presence of a few degenerate fibres.

4. In the peripheral stump Schwann cell activity begins on the 2nd day after cutting and from the 4th day rises rapidly to a peak at the 19th–25th day. It then falls quickly up to about the 60th day and afterwards more slowly. Activity is still appreciable more than a year after cutting. These changes of activity with time of degeneration are shown by thigh, knee and shank regions of the peripheral stump. The knee region, and the shank (which is 8 cm. distal to the initial cut) are more active than the thigh region, especially in the early days of degeneration.

5. In the central stump activity is at first confined to a few mm. immediately adjacent to the cut. From the 2nd to the 4th day after cutting the central stump is more active than the peripheral stump, but thereafter it is much less active. Its maximum activity, never, except for the bulb fibroblasts, more than 10% of the maximum activity of the peripheral stump, is reached probably between 5 and 10 days after cutting, after which it falls slowly. Activity is still appreciable more than a year after the cut was made. The more proximal part of the central stump, at first inactive, begins to show slight Schwann cell activity after 23 days and is still active after more than a year.

6. The $\frac{1}{2}$ cm. of the peripheral stump near the cut, including the bulb, is at first more active than the adjacent more distal region. But after degeneration of a year or more the peripheral bulb becomes on the contrary less active than the rest of the peripheral stump.

7. There is a particularly high fibroblast activity in the terminal bulbs of both peripheral and central stumps during the first 2–4 months, probably as a result of an early invasion from the neighbouring connective tissue. Relative to Schwann cell activity the bulb fibroblasts are most active during the 2nd–5th days of degeneration.

8. The schwannoma and neuroma in general show the same changes of activity as the bulbs from which they are formed. Almost no activity was found after degeneration of about a year. In the schwannoma no Schwann cells appear until the 6th day of degeneration, though fibroblasts are very active before this.

9. It is concluded that activation of the Schwann cells and fibroblasts is due to (*a*) degeneration of the nerve fibres, (*b*) in the region close to the cut, a traumatic effect of cutting the nerve, superimposed on (*a*).

10. Since Schwann cells probably play an important part in forming the junction when severed nerves are repaired this work indicates the optimum time from this point of view for making grafts or sutures. Our experiments indicate that the Schwann cells will be more active in joining together two nerve stumps if the nerve or nerve graft is left a few (say 10–20) days to degenerate before making the repair; and that (in the rabbit) the optimum time for suture is passed 25 days after the nerve is cut.

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OBSERVATIONS ON THE FORCES OF MORPHOGENESIS IN THE AMPHIBIAN EMBRYO

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(With Two Text-figures)

INTRODUCTION

Recent years have seen considerable advances in our knowledge of chemical interactions between different parts of developing embryos, and of the metabolic processes by which the stimulating evocators are released. We have also acquired further information, on the biological level, of the correlations between parts which lead to the formation of units organized into definite patterns. On the other hand, the forces which actually bring about the changes in shape which are perhaps the salient feature of early development have remained almost unstudied. Several hypotheses have been put forward. Thus some years ago, Glaser (1914, 1916) suggested that the folding of the neural plate into a groove is due to a weakening of the inner surface of the neural epidermis followed by an imbibition of water; the swelling consequent on this is supposed to extend to weakened inner surface more than the stronger outer one, so that the cells are deformed into truncated cones, and the whole layer folds. This conception has recently been criticized by Brown, Hamburger & Schmitt (1941) on the basis of accurate density determinations, which failed to reveal any evidence of imbibition. They suggest that the shape changes are primarily due to 'an increase in the "attractive" forces between molecules in the adjoining cell surfaces of prospective neural tissue cells so that the area of contact is actively increased'. Without making any hypotheses as to the physico-chemical mechanisms involved, Holtfreter (1939) has also emphasized the importance of attractive or repulsive forces dependent on the surface properties of the cells; in his case he was concerned with interactions between masses of tissue differing from one another in histological type. Finally, several authors (e.g. Harrison, 1936; Needham, 1936; Waddington, 1940) have drawn attention to the possibility that the facts could be explained if we could postulate the formation of submicroscopic fibrils (an orientated cyto-skeleton) within the cytoplasm.

The present communication presents data which bear on these possibilities from a number of angles. It had been hoped to record the observations in a more elaborate and statistical form, but as it seems improbable that the work can be continued in the immediate future, it has seemed best to make an interim report on the material, which, as far as it goes, is fairly straightforward and unlikely to be substantially altered by more quantitative study.

1. THE ORIENTATION OF YOLK GRANULES

At first sight the most obvious way of testing the suggestion that there is an orientated cyto-skeleton would be by the use of polarized light. Observations of this kind have been made on the chick embryo by Hobson (1941); and there are of course many studies on the eggs of invertebrates, where, however, the anisotropy has not in general been corre-

lated with form changes. In the amphibian embryo, this simple method cannot be satisfactorily used owing to the presence of large numbers of highly refractive yolk granules. These effectively obscure any double refraction which might be shown by the cytoplasm. The only recorded exception to this is in the long flask-shaped cells lining the early blastopore lip, where Waddington & Picken (cf. Waddington, 1940) observed a weak double refraction in the terminal processes, which are free of yolk, probably because they are too thin to contain the granules.

The presence of yolk granules, although it prevents the use of polarized light, can itself be made the basis of a method of studying a possible cyto-skeleton. The granules in most species of Amphibia are not perfect spheres, but are somewhat elongated ellipsoids. If there is a strongly orientated fibrillar structure in the cytoplasm, it would be expected that the granules would also be orientated with their long axes parallel to the fibrillar direction. This possibility has been investigated in a number of situations in which it might be expected to occur in embryos of *Triton alpestris* and *taeniatus*.

(*a*) *In the mitotic spindle*

It is becoming generally accepted that the mitotic spindle is a tactoid in which fibrous protein molecules are orientated in parallel (reviews by Darlington, 1939; Waddington, 1939*a*). The conditions are therefore such as should lead to the orientation of yolk granules. The spindle is, however, normally empty of granules, being formed of clear cytoplasm partly derived from the nucleus. Only rare cases of included granules have been found. In all these the orientation of the granules has been very exact, the long axis being parallel to that of the spindle. There seems no doubt therefore that yolk granules do become orientated if they lie in a region of cytoplasm which has a strongly fibrillar structure.

(*b*) *In the immediate neighbourhood of an elongating spindle*

The metaphase spindle in amphibian embryonic cells is rather short and broad. During anaphase it elongates considerably, pushing out in two directions through the yolky cytoplasm. If there was any pre-existing orientated skeleton in this cytoplasm, or if the cytoplasm was highly viscous, one would expect the elongating spindle to cause an orientation of the yolk granules in its immediate neighbourhood. This does not seem to occur. At metaphase the ends of the chromosomes protrude in a haphazard way into the cytoplasm, lying between and amongst the yolk granules. In spite of this, no evidence of orientation is visible in the granules immediately against the sides of anaphase spindles.

(*c*) *In the flask cells of the young blastopore*

In the two situations just mentioned, orientation might have been expected within a cell, but this would not necessarily have had any particular relation to morphogenetic developments. In this and the next two sections, evidence will be presented as to the orientation in cells whose shape is definitely connected with morphogenesis.

As has already been mentioned, the cells lining the early blastopore in the young gastrula become drawn out into long 'flask-shaped' ovals, with a thin neck reaching down to the blastopore from the thicker body which lies farther within the embryo. In the thinnest ends of the necks, no yolk granules are present, and the material has been shown to be anisotropic. In sections, however, the yolk granules within the body of the cells are found not to be orientated to a noticeable extent (Fig. 1*a*). The granules which

lie farthest down in the neck may, indeed, have their long axes parallel to that of the cell, but this seems only to be true when they are so narrowly confined that there is no room for them to lie in any other direction. Farther inwards, in the wider regions of the cell, the granules seem to lie completely at random. A full statistical analysis would be necessary to establish this strictly, but at least the appearances make it clear that any orientation which occurs is very much less than could account for the elongation of a more or less spherical cell into the long thin flask shape.

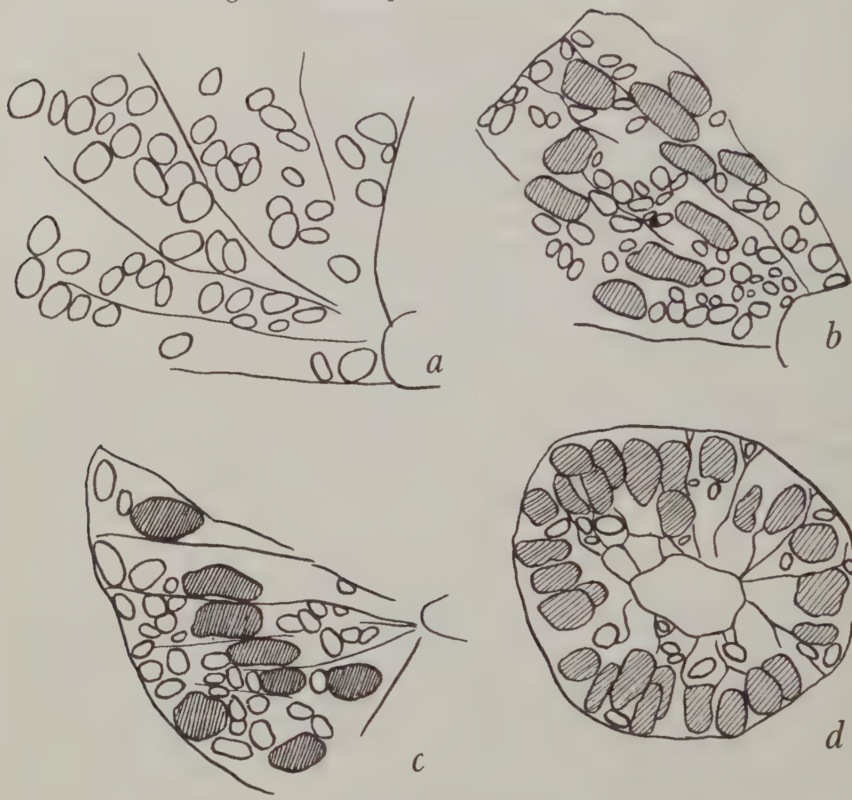

Fig. 1. *a*, Camera lucida drawing of the terminal ends of some 'flask-cells' extending down to the early blastoporal groove at the bottom right. Yolk granules indicated by hollow ovals, nuclei lie further towards the centre of the egg and are not visible. *b* and *c*, Similar drawings of groups of cells from the just closed neural tube; nuclei hatched. *d*, Lens vesicle shortly after becoming free from the corneal epithelium.

(*d*) *In the neural groove*

The changes of shape in the cells forming the neural plate, groove and tube have not yet been adequately described. Many authors have drawn attention to the elongation of the originally cuboidal cells of the ectodermal epithelium into a columnar shape, which then narrows on the outer surface to form a thin truncated cone. These changes do not, however, take place simultaneously over the whole area of the neural plate. In particular, the first change from a column towards a cone, which initiates the folding of the plate, occurs towards the edges of the neural area. The externally visible effect correlated with

this is the elevation of the neural ridges, outlining the still unfolded neural plate. It appears probable that a detailed study of the cell changes proceeding during these early stages in the folding would limit the types of hypothesis which could be advanced to account for the phenomena.

At present, however, we are concerned only with the orientation of yolk granules within the elongated columnar or conical cells. The granules in these cells appear to be in process of fairly rapid utilization, and they are not always easy to distinguish; the well-formed granules are accompanied by masses or more or less amorphous yolky material which is partially digested. The granules whose shape remains definite have been drawn in the figures, the other material being omitted (Fig. 1 *b*, *c*).

It is apparent that there is no noticeable orientation even in the very elongated cells of the early closed neural tube. In these cells the nucleus is usually long and oval in shape. Its long axis always lies parallel to that of the cell as a whole. It might be suggested that this is evidence that the cytoplasm in which the nucleus lies has an orientated structure. It is clear, however, that the cells are often so narrow that the nucleus could not be fitted in in any other way. The orientation of the nucleus can probably be more plausibly considered as a direct consequence of constriction due to the narrowing of the cell.

(*e*) *In the lens*

During the first stage of the formation of the lens in *Triton* the cells of the inner layer of the ectoderm elongate, forming a single-layered columnar epithelium. This then folds inwards, and becomes cut off as a small vesicle. Both in the columnar and the vesicle stages, the individual cells are considerably longer and narrower than in the original epithelium. No trace of orientation of yolk granules can be found (Fig. 1 *d*), except for such as are confined in very narrow spaces between cell surfaces; in such cases the orientation can be attributed to constriction by these surfaces rather than to a cytoplasmic micro-structure.

By the time the lens fibres are formed, the yolk granules have disappeared.

2. THE SHAPE OF CELLS IN MOVING CELL STREAMS

The investigation of early development by means of marks made with vital dyes has revealed the frequent occurrence of considerable translocations of cells by means of streaming movements. Very little is known about the physical forces involved in these movements, although preliminary measurements of their order of magnitude have been made by Waddington (1939*b*). Hypotheses as to the nature of the forces have to take account of the fact, recorded by many authors (e.g. Spemann, 1931; Waddington, 1942), that small packets of cells, transplanted into other regions, retain specific capacities to move in definite directions. This argues for the existence of some fixed polarity in the cells.

A conceivable basis for such a polarity would be a fibrillar organization of the cytoplasm, with orientation of the fibrils. A cyto-skeleton of this kind might have visible effects either on the orientation of the yolk granules or of the cell shape. Fixed and stained preparations of streaming regions of amphibian embryos have therefore been examined in an attempt to detect such effects; the material used was the roof of the primitive gut of a mid-gastrula and the overlying presumptive neural tissue (Fig. 2*a*, *b*). No evidence of any orientation of yolk granules could be detected. Moreover, as the

figures show, the cells are sensibly iso-dimensional, with no marked elongation in the direction of movement. Even the mitoses are not regularly orientated, and this is true not only in the tissue some distance away from the lip of the blastopore, but in the cells in its immediate neighbourhood, where the movement is most vigorous.

3. THE TENSILE STRENGTH OF THE CELL SURFACES

It is clear that alterations in the surface forces of the cells of different regions of the embryo, or of different parts of single cells, might produce changes in the shape of tissues or organ rudiments. No systematic attempts to detect such alterations seem to have been made. Almost our only source of information as to the surface forces in embryonic amphibian cells is the investigation of Harvey & Fankhauser (1933). They measured the tension at the surface of the newly fertilized egg of *Triturus* (*Diemyctylus*) *viridescens* by observing the flattening of the egg under the influence of gravity. The value

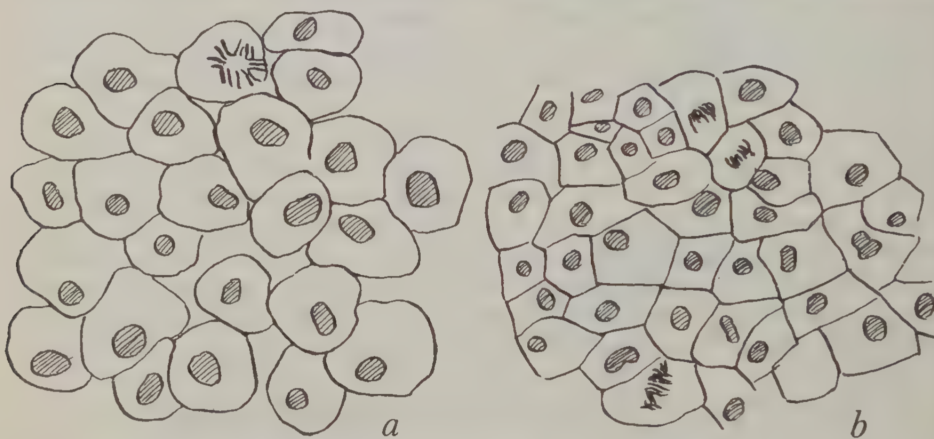

Fig. 2. *a*, A group of cells from the roof of the primitive gut of a mid-gastrula; they are moving in the direction of the top of the page. *b*, A group of cells of the presumptive neural plate overlying the last; the outline of the intercepts of the cells with the surface of the gastrula is indicated; the cells are moving towards the bottom of the page.

obtained was extremely low, less than 1 dyne/cm. Similarly, low values are typical of 'naked protoplasm' in general (cf. Newton Harvey, 1937). It cannot, however, be assumed that the surfaces of embryonic cells can always be considered to fall under the heading of 'naked protoplasm'. For instance, the newly fertilized eggs were studied by Harvey & Fankhauser immediately after they had secreted from the surface a fertilization (or vitelline) membrane whose strength is of quite a different order of magnitude. There is no reason why the cells should not in later development produce similar surfaces, whose strength might thus be considerably greater than that measured on the stripped egg.

Casual observations made during the normal operations of experimental embryology suggest that something of the kind may be true. Small fragments of tissue being transferred from place to place in pipettes sometimes come in contact with the water-air interface. Fragments of young embryos are immediately torn to pieces by the surface forces, but the tissues of much older embryos (late tail-buds and swimming stages) are

obviously tougher, and may be quite uninjured. This suggests a possible method of investigating the tensile strength of the cell surfaces.

Two methods have been used for testing the reactions of cells to applied surface forces. In the first, fragments of tissue were lifted by needles into the air-liquid interface of solutions of saponin, whose surface tension had been measured. In the second, the tissues were brought into the interface between water and various organic liquids. The embryos used are *T. taeniatus*.

After preliminary tests of a number of different saponin concentrations, solutions in tap water were made up of the dilutions 2×10^{-5}, 10^{-5} and 5×10^{-6} by weight. The surface tensions of these were measured by pulling a clean platinum wire through the surface. The values obtained (three measurements on each solution) were: solution 1, 50·4 dynes/cm.; solution 2, 55·8 dynes/cm.; solution 3, 60·6 dynes/cm. The tap water, measured at the same time, gave a value of 73·6 dynes/cm.

At the surface of all three solutions, naked newly fertilized eggs broke immediately and completely, as might be expected. In unfertilized eggs which had been kept at room temperature for 3 days, the surface had become extremely solid, and was not disrupted at the surface of any of the solutions or even of tap water.

Tissues of embryos ranging from young gastrulae to early tail-buds were studied. Tissues from all regions, in all stages, were broken on the surface of solution 3, with the highest surface tension. Differential behaviour was, however, shown with respect to the other two solutions with lower tensions. The ectoderm of the mid-gastrula was broken, though rather slowly, on solution 1, and rapidly on solution 2. By the early neural plate stage, the neural plate itself survived on solution 1, though the epidermis broke slowly. In the neural groove stage and later, both neural and epidermal tissues survived on this solution. Beginning in the open neural plate stage, a most interesting differential reaction of the neural tissue to solution 2 began to be manifested. If transverse sections of the neural tissue were carefully lifted into the surface, it was apparent that the inner surface of the neural tissue broke more readily than the outer. The difference was particularly marked in the neural groove stage, at which the inner convex surface broke down slowly while the outer concave surface survived uninjured. By the early tail-bud stage, neither surface of the neural tube was broken on solution 2, although the tension was sufficient to tear open the just-closing neural ridges, and flatten the tube into a shallow groove.

The epidermis was throughout slightly weaker than the neural tissue. The mesoderm was weaker still, but survived on solution 1 by the early tail-bud stage. The endoderm was the weakest tissue of all, failing on solution 1 even in the tail-bud stage. These observations are summarized in Table 1.

In using the second method, the interfacial tensions between tap water and the various liquids was not directly measured, but reference was made to physical tables (Landolt-Börnstein, 1912) where values of sufficient accuracy could be obtained. It was clear that the tissues used in these experiments were very rapidly injured by the quantities of the substances which dissolved in the water, and all tests were therefore made as quickly as possible; even so, probably no great reliance should be placed upon them, except as confirming the general picture emerging from the experiments with saponin solutions.

In general, tissues raised into the water-organic solvent interface broke at lower surface

tensions than those recorded for the saponin solutions. Thus all tissues tested broke on the water-carbon tetrachloride interface, with a tension of about 45 dynes/cm., although the neural tube of tail-bud embryos was only slowly disrupted. This neural tissue survived on the water-benzene interface, with a tension of about 35 dynes/cm., but other tissues (epidermis and endoderm) broke. Rather surprisingly, the epidermis was broken even on the water-chloroform interface (about 25 dynes/cm.), but this had no effect on the neural tissue or notochord of mid-tail-bud stages. The epidermis survived the lowest

Table 1. *Behaviour of tissues in solutions* 1 (*surface tension* 50·4 *dynes/cm.*) *and* 2 (55·8 *dynes/cm.*)

	Solution 1	Solution 2
Unfertilized egg fresh	Broke immediately	—
Mid-gastrula:		
Ectoderm	Broke slowly	Broke quickly
Mesoderm	Broke, surface of primitive gut going slowly	Broke quickly
Endoderm	Broke quickly	Broke immediately
Early neural plate:		
Neural plate	Survived	Lower surface broke more quickly than upper
Mesoderm	Broke slowly	
Endoderm	Broke quickly	
Neural groove:		
Neural tissue	Survived	Difference between surfaces very marked
Epidermis	Survived	Torn off mesoderm, but broke only slowly
Early tail bud:		
Neural tube	Survived	Torn open but cells not disrupted
Epidermis	Survived	Survived, but cut edges torn away from mesoderm
Mesoderm	Survived	Broke slowly

tension applied, that of the water-ether interface (about 11 dynes/cm.). At the tail-bud stage, the endoderm was the only tissue which could not support this tension, and it was only slowly disrupted. In the earlier stages, however (open neural plate and younger), all tissues were broken on this interface.

DISCUSSION

In the first two parts of this paper, phenomena were examined which might have been expected to reveal evidence of a fibrillar micro-structure of the cytoplasm. No such evidence appeared. The fact that yolk granules can be orientated in the mitotic spindle shows that such an orientation is possible in fibrillar surroundings. Its absence in the cytoplasm of cells undergoing changes in shape is therefore probably significant. At least it may be concluded that if any fibrillar cyto-skeleton is present, the forces exerted by the fibrils are much smaller than those in the spindle tactoid. It then becomes difficult to suppose that they can be strong enough to play any important part in bringing about the shape changes of morphogenesis.

The third part of the paper describes changes in surface phenomena which may be of more importance in this connexion. It should be said at the outset that the quantitative data are of an extremely rough nature. The surface of saponin solutions is known to be peculiar in many respects (Gaddum, 1932), since its tension depends on the length of

time it has been in existence and the rapidity with which it is stretched. The values for the surface tension given in the table were measured by a method which is not directly comparable with the phenomena which may be expected to occur when material is suddenly introduced into the surface, and therefore do not accurately represent the forces to which the embryonic cells were subjected. Moreover, it is possible that the saponin solutions had noticeable effects on the surfaces of the cells immersed in them; and this was certainly the case with the organic solvents used.

It can hardly be supposed, however, that the uncertainties in the measurements of the surface tensions of the saponin solutions affected the order of magnitude estimated, or the relative tensions of the different dilutions. The order of magnitude was almost a hundred times greater than the tensions measured in the naked surfaces of similar eggs by Harvey & Fankhauser. The greater strength of the cells of the later embryonic stages cannot be attributed to the effects of the saponin or the organic solvents, since these would be expected to diminish surface strength rather than increase it. It therefore seems justifiable to draw, from the present experiments, the first conclusion that the surface strength of cells in the gastrula and later stages is of an altogether higher order than that of 'naked protoplasm', being in the region of some tens of dynes per centimetre.

The second conclusion which seems justifiable is perhaps even more important. It is that the surface strength is not the same in all cells at all times. The evidence consistently indicates a gradual increase in strength throughout the gastrula to tail-bud stages. This affects all tissues, but not all equally. During these stages, the ectodermal tissues are always the strongest and the endodermal the weakest (in later stages the notochord seems rapidly to acquire considerable strength). Of the ectodermal tissues, the neural material is the strongest, and it shows behaviour which is perhaps the most interesting of all. The fact that the upper, concave, surface of the neural groove is stronger than the lower, convex, surface is exactly what would be expected if the surface tensions of the cells were responsible for the folding of the neural plate into the neural tube.

Although the evidence is thus in agreement with such a hypothesis, it cannot by any means be taken to prove it. In the first place, what has been measured is the passive strength of the cell surfaces against breakage by an applied force. In so far as the surface has any of the properties of a solid, this passive strength is not the same thing as the active force which the surface could exert to change the shape of the cell. In the second place, the greater strength of the concave surface might be a consequence rather than a cause of the folding. The folding, however it is brought about, is bound to involve a diminution in area of the cell surfaces abutting on the concave side, and this, for instance, by concentrating an ectoplasmic surface layer, might produce an increase in strength of these surfaces.

In the absence of any other well-established hypothesis, however, the suggestion that the folding of the neural tube is caused by changes of the surface properties of the cells remains an attractive one. The present investigation has been concerned only with the cell-water surface. In the embryo itself, forces arising in the cell-cell surfaces would also have to be considered. The relations between the forces in the two types of surfaces require much further study. Brown *et al.* (1941), who also tend to attribute neurulation to surface changes, have stressed the possibility that the primary force may be due to 'increases (in) the intercellular cohesion or "attraction", resulting in an increased area of contact between cells'. They show that the area of contact does increase. This would

also be expected as a result of effective contraction of the cell-water surfaces on the upper side of the neural plate, if this was brought about by an increase in their active surface tension. And there is, of course, no reason why cell-water tensions and cell-cell tensions should not both be concerned; in fact, one might suppose a priori that both types of surfaces would undergo simultaneous, though not necessarily similar, changes.

The discussion has so far tended to minimize the importance of fibrization as a factor in morphogenesis. This may be premature. Although the evidence strongly suggests that no importance can be attributed to fibrization within the body of the cytoplasm, the alterations which have just been discussed in the cell surfaces may well depend on the state of fibrillar aggregation of their protein constituents. Something of the kind, in fact, would seem to be the simplest way of accounting for the remarkable polar properties of cells such as those undergoing the invagination movements. Fibrization taking place in the cell surface might cause an increase in strength; and a similar orientation of the fibres in contiguous surfaces might account for the polarity. The positive evidence for such a suggestion is still meagre, but perhaps some support may be found in the observations of anisotropy in the thin terminal processes of the flask cells of the blastopore (Waddington & Picken, see Waddington, 1940). These thin ends consist mainly of the superficial ectoplasmic layer, and the fact that they show double refraction probably indicates that this layer contains orientated fibrils.

SUMMARY

1. The cells of early amphibian embryos contain ellipsoidal yolk granules. When a granule lies within the mitotic spindle, it is orientated with its long axis parallel to the direction of the spindle fibres. Yolk granules lying in the cytoplasm of cells are not specially orientated unless they lie in narrow spaces between cell surfaces; this is true in cells of the moving invagination streams, in the flask cells of the young blastopore, and in elongated cells of the neural groove and lens.

2. The breaking strain of cell surfaces was tested by bringing the cells into surfaces of known strength and observing whether they withstood the applied tension. The evidence indicates:

(*a*) That the breaking strain of cells from gastrula and tail-bud embryos is some tens of dynes per centimetre.

(*b*) That the breaking strain is not the same in all cells, being highest in neural tissue and lowest in endoderm during the stages mentioned.

(*c*) That the breaking strain gradually increases with age.

(*d*) That in the neural groove stage the strength of the outer concave surface of the groove is greater than that of the inner convex surface.

3. It is suggested that changes in surface tensions may be of importance in bringing about morphogenesis. The possibility is envisaged that the changes in tension may be connected with orientated fibrizations of the surface proteins.

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Note added in proof

Since the MS. of this paper was sent to press, I have had an opportunity of seeing the interesting discussion by F. O. Schmitt (*Growth Symposium*, 1942) of the importance of cell surfaces in morphogenesis. Many of the suggestions made there are similar to those advanced in this paper, and a stimulating discussion is given of the role of protein aggregates in cell-surfaces.

THE EXCITATION OF NEMATOCYSTS

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(With Three Text-figures)

Though an enormous amount of work has been done on nematocysts, the problem of how they are discharged remains unsolved. There are really two separate problems. First, what is the natural stimulus which excites the cnidoblast; second, what is the physical mechanism by which the nematocyst itself discharges? There is more work on the mechanics of discharge than on any other aspect of the problem, but it still awaits a satisfactory solution. As Graham (1938) points out, most of the work is remarkable for the chemical violence of the reagents used. Reactions to strong acids and alkalis, or to gross changes in osmotic or mechanical pressure can only throw an indirect light on the physical nature of the nematocysts' discharge. But it is not intended to discuss here the mechanics of discharge. Existing work has been reviewed by Weill (1934). This essay concerns the primary question of what is the effective stimulus to the cnidoblast, a problem to which much less work had been devoted.

There are two main hypotheses as to how the cnidoblast is excited. One suggests a mechanical and the other a chemical stimulus. Neither hypothesis completely covers the facts. Observation shows that cnidoblasts respond to contact. They must indeed be very sensitive, for Zick (1932) has shown in *Hydra* that a *Coleps*, $40 \times 20\mu$ moving 0·1 mm./sec. suffices to provoke discharge. Schulze (1922) suggested that the stimulus was essentially a mechanical one through objects touching the cnidocil. This might seem almost self-evident from the structure and distribution of these organs. Schulze interpreted the whole system as essentially mechanical. Bending of the cnidocil against the edge of the tube from which it arises, caused it mechanically to spring the cover of the nematocyst.

Schulze also showed that the structure of different cnidoblasts permitted a remarkable degree of differential response. In *Hydra*, prey possessing spines and bristles are mostly caught by volvent nematocysts which wind round the bristles. Organisms with flat surfaces are caught by the penetrants. He showed that this could be a mechanical consequence of the difference between the long thin cnidocils and wide cnidocil tubes in the volvents as compared with the corresponding structures in cnidoblasts of the penetrants. The thick cnidocils of the penetrants in their narrow cnidocil tubes were more easily bent to the edge of the latter, so that these cnidoblasts are the more sensitive and readily respond to pressure from a flat surface. On the other hand, any bristles covering the prey will sweep over the long cnidocils of the volvent cnidoblasts provoking their discharge.

But such mechanical devices fail to explain some essential features of the cnidoblast response. The discharge is selective. It does not generally take place to every solid object

but only to those of food value. Schulze himself showed that in *Hydra* the cnidoblasts are not stimulated by sand grains. This is a serious difficulty for the mechanical hypothesis. Zick (1929) pointed out an even more serious one. The commensal ciliates of *Hydra*, *Trichodina pediculus* (50–70μ) and *Kerona pediculus* (200μ), wander freely over the surface of the host bending the cnidocils right over, in fact much further than they are moved by the natural prey. Their ineffectiveness is not due to small size, since Zick (1932) shows that even such small prey as *Coleps* ($40 \times 20\mu$) suffices to excite the cnidoblast. It is thus evident that mechanical excitation of the cnidocil is not a complete explanation of a stimulus. Zick was reduced to the supposition that the cnidoblast response could be inhibited by the 'will' of the animal. But even this desperate assumption throws no light on the stimulus by which such inhibition could be produced. Moreover, in some coelenterates cnidocils seem to be absent (Weill, 1934).

The selective response of the cnidoblasts according to the nature of the stimulating object suggests that the stimulus may be a chemical rather than a mechanical one. The effect may be highly specific. Cardot (1927) showed in *Anemonia sulcata* that though the tentacles cling readily to the flesh of other species, they fail to discharge nematocysts into flesh of the same species. Nematocysts are easily excited by chemical stimuli. Glaser & Sparrow (1909) (*Physalia*) showed that they will respond to dilute acids and alkalis even when isolated. Apart from such drastic reagents which act directly on the cnidae, there are others that act by stimulating the cnidoblast. Parker (1905) remarks that KCl at 5 % or above added to sea water discharges the nematocysts of the lips of *Metridium*. But, KCl does not provoke a discharge in isolated nematocysts, so that we have here a chemical response by way of the cnidoblast to K ions.

Parker & Van Alstyne (1932), working on *Physalia* and *Metridium*, found that an extract of the skin of *Fundulus* caused the discharge of a limited number of cnidoblasts in situ, though without effect on isolated nematocysts. They pointed out that, contrary to the general opinion, mechanical stimuli by themselves are only very mildly effective. They boldly suggested that the effective stimulus to the cnidoblast is in fact a chemical one and that the cnidocil is a chemoreceptor. But if the stimulus to cnidoblasts were purely chemical, one would expect that food might normally activate them without actual contact. Such responses seem sometimes to occur, though only at such short distances as to render it possible that movement of the medium near the prey excites the responses (Zick, 1929, on *Hydra*). Moreover, Parker & Van Alstyne's food solutions never produced an intense discharge such as that which characterizes the direct contact of solid food with coelenterate tissue. Indeed, Parker himself (1916) found that the acontia of *Metridium* failed to discharge cnidae when flooded with juice from the flesh of *Fundulus*, and Zick (1932) showed that, although *Daphnia* is readily seized by *Hydra*, the latter does not discharge its nematocysts when flooded with an extract of these animals. Zick concludes that the natural stimulus cannot be a chemical one.

We thus come to a curious position. The hypothesis that the stimulus is mechanical explains many of the observations, but it fails to explain the vital fact that the response seems in some way to depend on the chemical nature of the food. On the other hand, the hypothesis of a chemical stimulus is not clearly supported by the effects of food solutions on the cnidoblasts, nor does it explain the apparent importance of direct contact in producing the response. In the following experiments this problem has been investigated chiefly by studying the cnidae of *Anemonia sulcata*.

THE CNIDOBLASTS OF ACTINIANS

The tentacles of *Anemonia* are thickly set with cnidae which discharge into the prey during feeding. As is usual among actinians, the cnidae are of two kinds. Besides the nematocysts there are the peculiar adhesive spirocysts. The cnidae are most easily examined in the following way. Cover-slips are coated with a film of saliva and allowed to dry thoroughly. A tentacle is then cut off the animal and allowed to touch the cover-slips. This causes an abundant discharge of cnidae on to the cover-slip, particularly where there is any aggregation of organic matter. The cnidae are easily stained with 1 % methylene blue to show the nematocysts and 1 % acid fuchsin to show the spirocysts (Stephenson, 1929). The nematocysts present are spirulae; the basitriches of Weill (1934). They are abundant and usually are evenly distributed over the cover-slip. In form they are like those from the acontia of *Phellia gausapata* (Stephenson, fig. 1 K, p. 177). The size of the nematocysts is about $27 \times 2{\cdot}6\mu$.

Abundant spirocysts are also present forming tangled clumps over the cover-slip. The filaments are long, and in the undischarged spirocysts they are neatly and tightly coiled in a characteristic manner. Their size is from 20 to $27 \times 3{\cdot}0\mu$. Both nematocysts and spirocysts fire off into pieces of food but not usually to inert substances such as glass cover-slips, provided these are absolutely clean.

In all anemones the very numerous nematocysts and spirocysts are contained in long narrow cnidoblasts, which they almost fill. It has not yet been shown whether these are surmounted by cnidocils. Parker & Van Alstyne (1932) speak of abundant 'cnidocils' on the tentacles of *Metridium*; but there are no stout stiff bristles like those of the Hydrozoa in this or in any other actinian so far as is known. Iwanzoff (1896) states that in *Anemonia sulcata* and other actinians he observed that one of the cilia borne by the cnidoblast was transformed into a fine and delicate cnidocil. But he says this 'cnidocil' is hardly perceptible, and, as Weill (1934) points out, no one has confirmed this observation.

Parker & Van Alstyne probably refer to the numerous conical refractile projections scattered over the ectoderm of most actinian tentacles. These were first described by v. Heider (1877) who said they were cnidocils. They were afterwards carefully studied by the Hertwigs (1879). These authors found that a fine stiff cilium protrudes from some of the cones. They considered that these cones were probably sensory structures, but that cones without the stiff cilium probably corresponded to cnidocils. However, they arrived at no definite decision, and indeed it is a difficult point to settle. Weill (1934) in his great monograph does not discuss these possible cnidocils. He declares he has never seen a cnidocil in any anthozoan and that all the ectoderm cells, cnidoblasts included, have only functional cilia or an irregular 'brush border'.

The Hertwigs showed that the cones are compound structures made of fused cilia, into which components they are easily disintegrated. They are easily seen in the living tissue (Fig. 1*a*) or when freshly fixed in 1 % osmic acid in sea water. But they rapidly disintegrate under bad conditions and do not survive most fixatives. My observations were made on the tentacles of *Anemonia sulcata* and *Metridium senile*, either living in Mg sea water or fixed chiefly in 1 % OsO_4 (in sea water or $0{\cdot}6M$ NaCl) for 20 min. followed by washing in $0{\cdot}6M$ NaCl and post-fixation in Bouin's picro-formol-acetic.

In general my observations confirm those of the Hertwigs. There is evidence that some of the ciliary cones are carried by sensory cells. Gentle stroking of the cones produces a

reflex contraction of the tentacular muscles. Sections show that some cones are borne by elongated deeply penetrating cells (Fig. 1*b*). These can also be seen in living tissue. If a tentacle is kept for 20 or 30 min. under a cover-slip many of the ectoderm cells become highly vacuolated and, in the process, the crowded cells of the ectoderm become separated

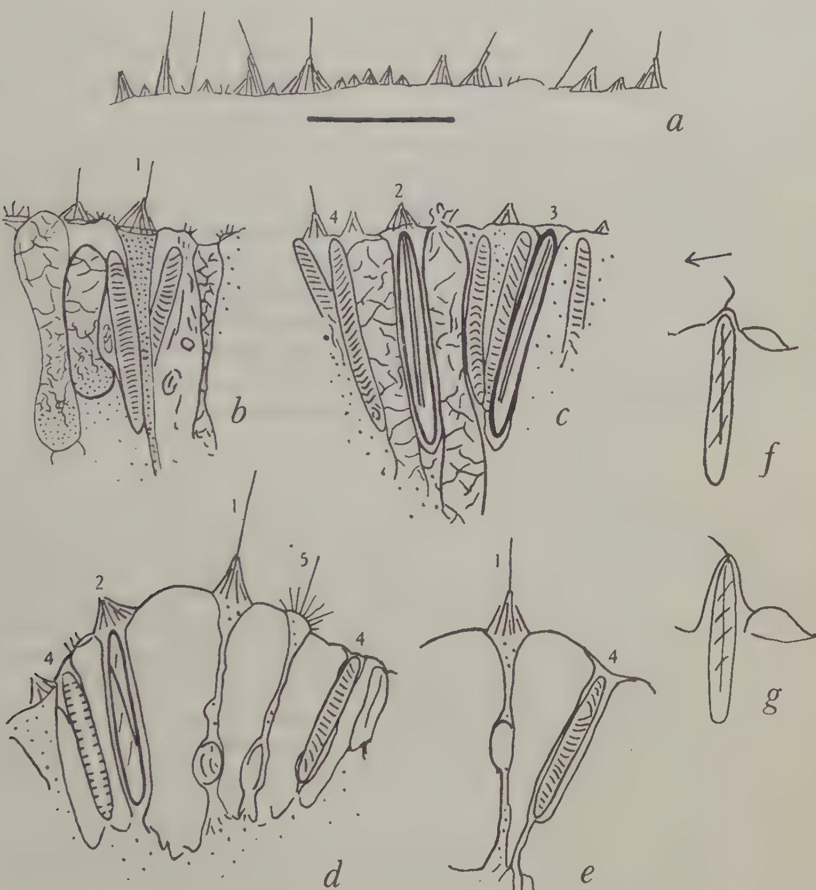

Fig. 1. *a*, edge of living *Anemonia* tentacle showing ciliary cones. *b* and *c*, 4μ sections of *Anemonia* tentacles fixed osmic and Bouin: showing ciliary cones over 'sensory' cell (1) and over nematocyst (2), and showing nematocyst (3) and spirocyst (4) not surmounted by ciliary cones. *d* and *e*, optical sections of living *Anemonia* tentacles after 30 min. Vacuolated ectoderm separating various types of cell: (1) 'sensory' cells beneath ciliary cones, (2) nematocysts beneath ciliary cones, (3) coneless nematocyst, (4) spirocysts without cones, (5) 'sensory' cell with cone disintegrated into cilia. *f* and *g*, living tentacle of *Metridium*. Successive stages in the extrusion of a nematoblast from the ectoderm, bearing a single active cilium. Bar $= 20\mu$.

(Fig. 1*d*). The long cells beneath some of the ciliary cones, particularly those carrying a stiff cilium, are easily seen.

The dense packing and distortion of the elongated ectodermal cells make it difficult or impossible to prove by the examination of sections that a particular ciliary cone or other structure belongs beyond all possible doubt to a particular cell. But both in sections

and in living tentacles undergoing vacuolation there seems to be direct evidence that some nematoblasts bear ciliary cones (Fig. 1 *c*, *d*). On the other hand, many apparently normal cnidoblasts clearly do not possess them, and I have never seen spirocysts which bore them for certain (Fig. 1 *c*, *d*, *e*). Many nematoblasts bear no special structures on the outer surface. Some, however, bear a single cilium as can be seen beyond doubt when the nematoblast happens to be extruded carrying the cilium on its end (Fig. 1 *f*, *g*). These cilia may be intermittently active or apparently permanently at rest in the upright position. It may have been these that gave rise to Iwanzoff's observation of fine 'cnidocils'.

Though therefore some cnidoblasts apparently bear ciliary cones, many others do not. Moreover, it can be shown that cnidoblasts possessing no cones can be fully functional. In some individuals cones are much less developed than in others. In any one individual there are fewer at the base of the tentacles than at the tip, and they are least developed in the outermost cycles of the tentacles. Particularly in *Metridium*, these parts may be visibly devoid of cones. Yet touching these parts with an active solid, such as a human hair, may produce as good a cnida discharge as touching the regions bearing cones.

It seems therefore that no special cnidociliary structures are necessary for the normal functioning of the cnidoblasts, though some of the latter may possess single cilia, or cones of fused cilia like those carried by other, probably sensory, kinds of cell.

CNIDAE AS INDEPENDENT EFFECTORS

The first question to consider is whether the cnidae are under the control of the nervous system. If so, their discharge might be controlled by many distant sense organs responding to different stimuli. The balance of the histological evidence is against the presence of nerves running to the cnidoblasts (Parker & Van Alstyne, 1932; Weill, 1934). The physiological evidence points fairly clearly to the cnidae being independent effectors as Parker & Van Alstyne (1932) show. It is not possible to obtain a cnida response by the conduction of excitation from a distant stimulus. A normal food object touching a tentacle of *Anemonia* only provokes a discharge at the point of contact. Stimulation by inert objects not only produces no response but also in no way reduces the cnida response to solid food when this is allowed to touch adjacent regions of the tentacle. There is neither conducted excitation nor inhibition.

Similar evidence is obtained from electric stimulation. Parker & Van Alstyne found that the cnidae of the acontia of *Metridium* and of the tentacles of *Physalia* were very easily discharged by induction shocks sent in through micro-electrodes. The discharge was always confined to the immediate region of the stimulus and was not conducted. I have performed similar experiments with the isolated tentacles of *Anemonia sulcata*. A tentacle was placed under a few mm. of sea water in a Petri dish under the microscope. It was stimulated by Ag-AgCl-sea-water electrodes. The anode was an Ag-AgCl plate along one edge of the dish. The cathode was contained in a glass tube filled with sea water and placed on the opposite side of the tentacle. The opening of the cathode was 50μ in diameter and it was placed 120μ from the tentacle. Condenser shocks were then passed through the system from a 2μF. condenser charged to 240 V and discharging through a 400 Ω potentiometer whence the electrode leads were taken. The response showed a definite threshold. Different adjacent regions of the tentacle stimulated with the cathode the same distance away gave thresholds which varied ± 10 %. The results fully confirmed

Parker & Van Alstyne. The discharge of the cnidae was always quite local. There was no evidence of conduction even with strong shocks. Moreover, these experiments proved that the stimulus was truly electrical, for the use of non-polarizable electrodes ensured that the stimulation of the cnidae was not due to any chemical influences caused by electrolysis.

Discharge of the cnidae fails to spread not only with single shocks but also with series of shocks sent in at various frequencies. This is important because such repetitive stimulation causes a rapid spread of excitation in the nerve network suplying the muscles, even to distant parts of the tentacle (Pantin, 1935). At no frequency of stimulation does spread of excitation in the nerve net result in excitation of distant cnidae. There is thus no physiological evidence that the cnidae are connected with the nervous system, nor is there evidence that excitation can in any other way be conducted to distant cnidae.

Although the nervous system does not play a part in the discharge of the cnidae, the cnidoblast certainly contains excitable elements. Like other excitable cells they are anaesthetized by Mg salts. Solutions of half 0·6 m./$MgCl_2$ plus half sea water, 'Mg sea water', prevent the cnida discharge in *Anemonia* tentacles about as quickly as the muscular response of the tentacles is prevented, a matter of a few minutes. Though therefore the cnidoblast behaves as an independent effector, its excitability to electric stimuli and the influence of Mg ions show that it has its own excitable system, and that its discharge is no mere mechanical process due to movement of a cnidocil, even if such were present.

MECHANICAL EXCITATION

Mechanical responses of the cnidae are easily tested in the following way. Isolated tentacles of *Anemonia* are placed in sea water under the microscope. If a hair or a small piece of human skin is mounted on a fine glass needle it can be brought to touch the tentacle and the discharge of the cnidae observed. It becomes immediately apparent, first, that the discharge only takes place where the hair or skin touches the tentacle. Secondly, the discharge into the hair or skin is complete. Even a hair 100μ across may receive such a potent discharge of nematocysts and spirocysts that the tissue of the tentacle is pulled outwards when the glass rod is withdrawn, which may even suffice to drag the tentacle along (Fig. 2*a*). This shows clearly that tactile stimulation is of fundamental importance in the normal discharge of the nematocysts.

Though the immediate stimulus is tactile, it normally only produces a response when the object is of food value. A small capillary bead (100–500μ) at the end of a glass rod will, if chemically clean, produce no discharge (Fig. 2 *b*, *c*), though a very little handling with the fingers suffices to make it active. Even so, the lack of responsiveness to clean inert surfaces is only relative. In 'hungry' anemones that are in good condition some discharge may be made even to a clean capillary ball. Even with normal *Anemonia* tentacles there is considerable individual variation of sensitivity. In occasional animals the nematocysts are discharged on to inert surfaces, while at the other extreme a few animals, particularly those that have been well fed, show no discharge even into food particles. In any one case, however, the discharge on to food particles vastly exceeds that on to inert substances and requires far less mechanical disturbance.

The extent of the mechanical response is thus a question of degree. Even for the tentacles of average animals it is possible to obtain graded discharge to clean glass surfaces according to the extent of the mechanical stimulus. As we have said, if a tentacle is

touched by a smooth capillary ball no response from the cnidae is obtained. But if a capillary rod is broken so as to give a sharp surface, violent scratching of the tentacle with this cut surface produces a discharge of some nematocysts, though not of many. Finally, if the tentacle is exposed to a maximal mechanical stimulus by lifting it out of

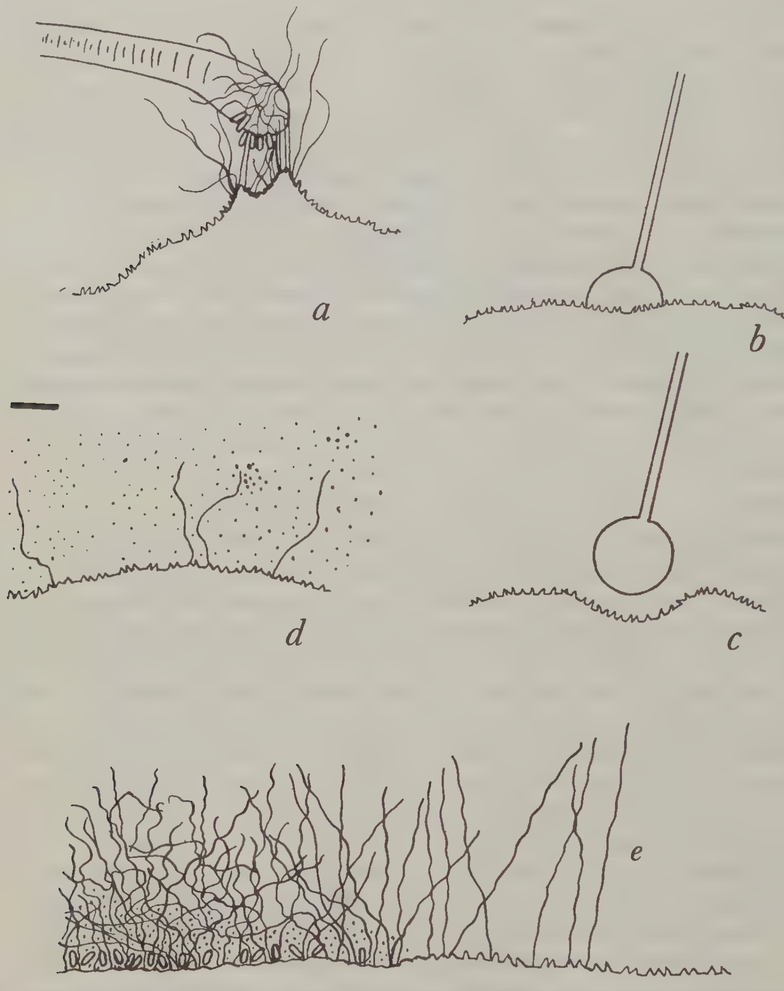

Fig. 2. *Anemonia sulcata* tentacles in sea water. Bar = 100μ. *a*, response of cnidae to touch by human hair. *b* and *c*, lack of response to clean glass bead. *d*, response to immersion in 1 % dry weight of human saliva in sea water. *e*, response to drop of 1 % Na taurocholate diffusing along tentacle from left to right.

the water and then dragging it along a clean dry glass surface, a considerable discharge is often obtained. This shows that discharge of cnidae depends on the degree of mechanical stimulation. With food the threshold is low, with inert substances it is high. There is no doubt that with inert substances a purely mechanical stimulus at times suffices, though with difficulty.

The mechanical stimulus is affected by the mucus secreted by the anemone tentacles.

A glass rod or even a food particle which has become covered with the anemone's own mucus is quite ineffective in producing a response from the cnidae. The discharge on a dry plate does not take place with tentacles covered with a thick mucus layer. This effect of mucus may possibly be due to some chemical influence, but examination under the microscope suggests strongly that the effect is due to reduced mechanical stimulation. This is rendered more probable because while anemone mucus on the food may reduce the response the mucus layer over the tentacle may sometimes increase it. When a food particle has caused a small local discharge of cnidae on to it there is also a local secretion of mucus by the tentacle. If the food particle is now moved about in an endeavour to remove it from the tentacle, the mucus in the immediate neighbourhood is violently moved and this may cause a further local discharge of cnidae.

CHEMICAL EXCITATION

Cnidae are easily discharged by chemical action. In *Anemonia*, sea water diluted to half strength or brought to *p*H 2·0 or below by HCl causes immediate discharge. It is certain that such powerful reagents are not the normal stimuli to the cnidoblast. They act equally well on isolated cnidae as on the cnidoblasts of the living tentacle. Food, on the other hand, causes no response in isolated cnidae. Moreover, the response to strong reagents is unaffected by 'Mg sea water' which rapidly paralyses the natural response of the cnidoblast to food objects.

Sea water made less acid than *p*H 2·0 with HCl does not cause a discharge. Below *p*H 4·0, however, it causes the majority of the cnidoblasts to extrude the cnidae in the unshot condition. The significance of this response is not clear, but it is a frequent one in irritant solutions. It is characteristic of the effect of flooding *Anemonia* tentacles with a solution of ether in sea water, 2 % triacetin, or quinine at or above 0·1 %, and in other cases to be mentioned later. The extrusion is always accompanied or perhaps caused by great muscular contraction and secretion of mucus. Extrusion, contraction and mucus excretion are all inhibited by 10 min. immersion in Mg sea water. Extrusion is not a normal accompaniment of the response to food.

There are few records of dissolved substances which excite the cnidoblast to discharge its cnida but which have no action on isolated cnidae. Parker (1905) noted that the cnidae of the lips of *Metridium* were discharged by KCl added to sea water. Potassium is certainly a powerful agent for the activation of cnidoblasts in some anemone tissues, and it produces no direct effect on unshot isolated cnidae. Like those of the lips of *Metridium*, the cnidoblasts on the acontia of *Calliactis parasitica* are highly susceptible to the action of potassium. Even as little as 5 % of 0·6*M*/KCl (isotonic) added to sea water suffices to shoot many of the cnidae, while greater concentrations completely discharge every cnida in the acontium. On the other hand, in the tentacles of *Anemonia* 5 % 0·6*M*/KCl (isotonic) added to the sea water produces little effect other than slight muscular activity. At about 10 % 0·6*M*/KCl, there is a characteristic contraction of the musculature. At 20 % 0·6*M*/KCl there is, in addition, extrusion of a fair number of cnidae, though the majority of these are not shot. At 40 %, very large numbers of cnidae are extruded, some of which are shot, but by no means the majority. The violent contractions induced in the tentacle by strong KCl suggest that in this case the action of potassium on the cnidoblast may be of mechanical rather than chemical origin. Though it is significant that some cnidoblasts like many other excitable cells are stimulated by

excess potassium, the concentration required to stimulate the cnidae of *Anemonia* tentacles is far above that which could normally diffuse from a food object.

Parker & Van Alstyne's hypothesis of chemical activation of the cnidae is derived from the observation that an extract of the skin of *Fundulus* causes a limited spontaneous discharge of cnidae. In *Anemonia* tentacles, food solutions do not in general cause any spontaneous discharge. In certain cases, however, high concentrations of food solutions caused a few scattered cnidae to discharge spontaneously (Fig. 2*d*). This is true of the following solutions in sea water, by dry weight: 10 % egg albumen, 10 % Witte's peptone, 0·5 % human saliva (equivalent to 50 % wet weight of saliva). Even in these cases one cannot be certain that the stimulus to discharge is purely chemical. Strong food solutions cause writhing contractions of the tentacles, and cnida excitation might be due to mechanical stimuli originating in this way. In such experiments care must be taken that no solid particles are present in the solution, otherwise these may provoke a discharge.

The inability of food solutions to cause any well-marked discharge of cnidae is shared in a striking way by most food derivatives. We shall show elsewhere that the muscular responses by which the tentacles convey food to the mouth are excited through the agency of specific chemical substances, notably proteins and their derivatives such as amino acids. But amino acids do not cause any spontaneous discharge of the cnidae in the tentacles of *Anemonia*, nor do they exert any other effect upon the cnidoblasts. Table 1 shows that sugars and glycerol are also without effect.

Table 1. *Substances without action on the cnidoblasts*

Saturated solutions:	Asparagine	Aspartic acid	Creatine	Cystein
	Cystine	Histidine	Leucine	l. Tryptophane
	Tyrosine			
10 % solutions:	Arginine	Glutamic acid	Glycine	Proline
	Glucose	Lactose	Laevulose	Maltose
	Saccharose			
	Glycerol			

For the more insoluble amino acids saturated solutions were prepared. Enough dilute acid or alkali was added to increase the amount dissolved without carrying the reaction of the solution beyond the range *p*H 7·0 to 9·0. The response of the cnidoblast to solid food is not influenced by the *p*H of sea water within this range. The more soluble amino acids were made up as 10 % solutions at about *p*H 8·0. Even higher concentrations were without effect, but their very high osmotic pressure made the stimulus abnormal.

Although it has no action on the cnidoblasts, glycine strongly stimulates the tentacles. A 10 % solution in sea water causes writhing contractions with the production of much sticky mucus so that a glass bead sticks to the tentacle; but there is no discharge of cnidae.

Though normal food derivatives have no obvious effect on the cnidoblast, certain other substances were found to be very active in causing a discharge when tentacles were flooded with them. The lower fatty acids cause a spontaneous discharge of the cnidae in solutions that are far less acid than is the case with mineral acids. Acetic (C_2), butyric (C_4), caproic (C_6) and caprylic (C_8), all produce spontaneous discharge in concentrations at and above 0·1 %. The *p*H of such solutions ranges from *p*H 4·0 to 4·6. Beyond caprylic acid, the fatty acids are too insoluble to be tested in this way. Though all the soluble fatty acids are more effective than HCl, there is no significant difference in effectiveness between different members of the series.

The action of such fatty acid solutions differs from those of mineral acids in one

important particular. They have no effect on isolated cnidae, and their effect on the cnidoblasts in the tentacles is easily paralysed by Mg. Even 5 % butyric acid in Mg sea water produces no discharge of cnidae. These acids therefore act by stimulating the cnidoblast. The effective agent appears to be the undissociated fatty acid. 1 % solutions of the acids cause complete discharge, but if the solutions are brought back to *p*H 8·2 by the addition of NaOH, the resulting solutions of the sodium salts are without effect.

Like the acids, the corresponding aliphatic alcohols excite the cnidoblasts to discharge, though they are very much less effective. Other substances of very different kinds cause spontaneous discharge. Quinine chloride 0·1 % in sea water *p*H 8 causes a slow spontaneous discharge in the tentacle. But by far the most effective agents in exciting the cnidoblasts to discharge are the bile salts and saponin (Fig. 2*e*). A 1 % solution of saponin or sodium taurocholate in sea water excites the discharge of every cnidoblast in the tentacle. Flooding a tentacle with such a solution makes a very pretty method of observing the mode of discharge of the cnidae, although—as we shall see—it only gives indirect evidence as to the normal method of activation. Table 2 shows the substances found to excite the cnidoblasts to spontaneous discharge. It is noteworthy that all these substances are cytolytic agents. Solutions at threshold concentration for excitation of the cnidoblast gradually produce cytolysis of the whole tentacle if immersion in them is continued for ½ hr. or so.

Table 2. *Substances exciting cnidoblasts to spontaneous discharge. Threshold concentration*

Acetic acid, Butyric acid, Caproic acid, Caprylic acid	0·1 %, *p*H 4·0–4·6
Ethyl alcohol	3–10 %
Isopropyl (C_3) alcohol	3–10 %
Butyl (C_4) alcohol	3 %
Amyl (C_5) alcohol	2 %
Quinine	0·1 % (spontaneous discharge slow and incomplete)
Saponin	0·1 %
Na taurocholate, Na glycocholate	0·1 %

These experiments show that cnidoblasts are excitable by certain chemicals. Chemical substances of a similar character might conceivably be present in foods. Could chemical excitation by the diffusion of such substances be a normal cause of the response of cnidoblasts to food objects? Flooding of a tentacle with 1 % bile salt causes prompt discharge of cnidae freely into the medium. But if a solid object is subsequently brought in contact with the tentacle it fails to adhere to it, because the cnidae are already shot. Whether the stimulus is chemical or not, the cnidae are only effective if they are shot at the same moment that the prey makes contact with the cnidoblasts.

The importance of this can be shown in the following way. Pieces of clean cotton-wool of about 2 ml. in volume are taken. If one of these is soaked in sea water and then allowed to touch a tentacle of *Anemonia*, no cnida discharge is provoked such as would have been the case to a lump of solid food. If a similar piece of cotton-wool soaked in a food solution such as 0·2 % (dry weight) of beef in sea water touches a tentacle, this instantly adheres strongly to it owing to the discharge of cnidae in contact with the cotton-wool. But if another piece of cotton-wool is soaked in 1 % bile salt, there is no

adhesion when it comes in contact with the tentacle. Examination shows that the cnidae are discharged at a distance before the cnidoblasts actually touch the cotton-wool.

This experiment shows the importance of the discharge taking place simultaneously with contact by the food object, if it is to be effective. If food normally excites the cnidoblasts by direct chemical action after the manner of bile salts, there must be some special mechanism to ensure that the threshold for chemical excitation was exactly reached at the moment contact was made. This condition can only be artificially fulfilled in the case of bile salts by using a threshold concentration (0·1 %) with which to soak cotton wads. The tentacles do in a few such cases cling weakly to the wads. But all higher concentrations are ineffective through premature discharge—unlike food solutions which only occasionally produce spontaneous discharge in the highest concentrations.

There can be no doubt that chemical stimulation is in some way involved in the cnida response, but whatever part it may play chemical excitation alone cannot account for the response of the cnidae to food.

EXCITATION BY FOOD

During the experiments with bile salts it was sometimes found that solutions with a concentration (0·01–0·03 %) definitely below the threshold for spontaneous excitation of cnidoblasts, could activate cotton wads so that these provoked a weak discharge of the cnidae when they made contact with a tentacle. Though only imperfectly shown with bile salts, this effect was found to be strongly characteristic of food solutions, such as beef extract, human saliva and the mucus secretion of molluscs. The relative effects of some food solutions and surface active substances are shown in Table 3.

Table 3

% dry wt.	Discharge: Spontaneous	Discharge: To touch
Saliva: 0·5	⊕	+++
0·1	O	+++
0·03	O	+
0·01	O	O
Egg white: 7	⊕	++
2	O	++
0·2	O	O
Witte's peptone: 10	⊕	+++
1	O	+++
0·3	O	+
0·1	O	O
Na taurocholate: 1·0	+++	(Shot already)
0·1	+++	,,
0·03	O	O or +
0·01	O	O
Saponin: 10	+++	(Shot already)
1·0	+++	,,
0·3	++	,,
0·1	+	,,
0·03	O to ⊕	O or +
0·01	O	O

Concentrated food solutions sometimes cause limited spontaneous discharge. But far lower concentrations are strongly effective in producing a discharge to cotton-wool. Thus saliva at 0·5 % dry weight is required to produce any spontaneous discharge which in any case is not great. But the tentacles adhere to pieces of cotton-wool soaked in

saliva as dilute as 0·01 % dry weight. Similarly, though Witte's peptone produces a little spontaneous discharge above concentrations of 10 %, the tentacles will adhere to wads soaked in concentrations of it as low as 0·3 %. In all such cases the tentacles of the anemone can be seen to discharge their cnidae on to the wads where contact is made.

These experiments suggest that the chemical stimulus plays a different role in the discharge of the cnidae from that usually supposed. This can be conveniently studied by observing under the microscope the response of the cnidoblasts to contact with a small glass bead (100–500μ) while immersed in sea water containing different substances in solution. We have already seen that solutions of sodium taurocholate above 0·1 %, etc., causes a general discharge of the cnidae. If tentacles immersed in such solutions are touched by the glass bead there is no adhesion to the discharged cnidae or to the tentacle itself. Previously discharged cnidae will not adhere to food. It is only when the discharge takes place directly with the solids in contact with the cnidae that it is effective.

It has been pointed out that a food response can sometimes be obtained to cotton-wool wads soaked in taurocholate solutions just below the threshold of chemical excitation. If the response of the cnidae in such solutions is examined by touching the tentacle with a glass ball, it is found that in those cases where the dilute taurocholate is effective in producing a feeding response, there has in fact been little or no spontaneous discharge of the cnidae but that the responsiveness of the cnidae has nevertheless in some way been heightened so that they discharge more easily to touch with the glass ball than they would in plain sea water. With taurocholate this effect is only seen near the threshold, and a tenfold increase in concentration merely causes spontaneous discharge into the solution. But this gives the clue to the true feeding reaction of the cnidae.

We have shown that there is a normal discharge to contact with cotton-wool wads soaked in food solution, even when these are too dilute to cause any spontaneous discharge of the cnidae. Under the microscope this is a very instructive experiment. Two isolated tentacles of *Anemonia* are taken: one is immersed in ordinary sea water, the other in sea water to which has been added 0·1 % dry weight of saliva which has been centrifuged to remove solid particles. After a few minutes the response of the cnidae in the two tentacles is tested by touching with a clean glass bead. The cnidae of the tentacle in ordinary sea water show no response whatever to mechanical stimuli by the ball. But the tentacle in the presence of the saliva has radically changed its behaviour. There is no visible spontaneous discharge of cnidae, but the slightest mechanical disturbance of the surface of the tentacle by the clean glass ball now produces a large discharge of cnidae on to the ball. The effect of the food solution has been both great and indirect. It has not directly stimulated the cnidae but it has enormously lowered the threshold to discharge by mechanical excitation (Fig. 3*a*).

This type of reaction is characteristic of natural food solutions at all concentrations. Though surface-active substances may produce a similar effect just at the threshold concentration, a small rise in concentration suffices to cause a spontaneous discharge. With food solutions the concentration may be raised a hundred times above the threshold without interfering with the primary effect of sensitization to mechanical stimuli. Even the apparent spontaneous discharge which takes place in strong food solutions may really only be due to heightened mechanical sensitivity, for it is characteristic for such solutions that they send the tentacle into violent writhings and contractions.

These experiments show that the stimulus for the cnidae is essentially a double one.

The immediate stimulus is a mechanical one, but it is only effective if the threshold is lowered by the presence of certain substances present in natural food. The time relations of the two components of the stimulus are very different. The mechanical stimulus is optimal for disturbances lasting only a fraction of a second. The chemical effect is much more enduring. The sensitivity of a tentacle exposed to 10 % saliva continues to increase for 4 or 5 min. and the effect may last for 30 min., that is, long after adaptation to chemical stimuli by the ordinary neuromuscular system.

The cnidoblast is therefore a unique tissue element. As an independent effector it contains sensory, excitor and effector elements. But the sensory element is itself not simple and performs the duties discharged in the neuromuscular system by two distinct sense organs, mechanical and chemical. The cnida may in fact be said to be a double sense organ as well as an effector. There are no obvious analogies to this in the tissues of the higher animals.

Fig. 3. *Anemonia sulcata* tentacles. Bar = 100μ. *a*, sensitization of cnidoblasts to glass bead by 5 min. immersion in 0·1 % dry weight of saliva in sea water. *b*, response to glass bead smeared with alcoholic extract of *Pecten* mantle.

THE ACTIVE SUBSTANCE

Natural food lowers the threshold of the cnidoblasts to mechanical stimuli. To what constituents is this probably due? Table 4 shows the relative sensitizing effect of a number of foods and of their derivatives. It also shows their relative effect on the sense organs of the tentacles as shown by their neuromuscular response in the feeding reaction. In general, the same foods activate both the cnidoblasts and the tentacles. Certain foods rich in protein are especially effective in both cases, notably natural mucus secretions.

It will be shown in another paper that the sense organs of the tentacle are particularly sensitive to pure proteins and to their derivatives, peptides and amino acids. This, however, is not true of the cnidoblasts, because purified proteins lose their effectiveness —even in the case of mucin itself. This is particularly true of fat-free protein. Amino acids themselves do not sensitize the cnidoblasts even in concentrations of 10 % or more (Table 2). Therefore, though the same foods activate the feeding movements of the tentacles and sensitize the cnidoblasts, the latter are responding to some constituent other than protein derivatives. Table 4 shows that this constituent cannot be a sugar, though it might be related to fats. Although it is not a protein, it is closely associated with proteins. Prolonged washing of a suspension of beef in distilled water by repeated centrifuging and resuspension does not greatly reduce the power of the centrifuged flocculum to discharge

the cnidoblasts on touch. Again human saliva or beef suspension (4 % dry weight), which are highly active on cotton wads, were subjected to ultrafiltration through collodion membranes under a pressure of 40 mm. of mercury. The clear, protein-free ultrafiltrate had no sensitizing effect. The active substance is thus either colloidal or adsorbed on the protein or other colloids present.

Experiments with suspensions of beef showed that the active substance is closely associated with the fate of the protein. After prolonged boiling the coagulum from a 4 % beef suspension is highly active on touching a tentacle, while the clear filtrate is without sensitizing effect. Similarly, the coagula from beef suspensions made acid to *p*H 4·5 with acetic acid were strongly active, whilst the neutralized filtrate had no sensitizing effect. Some even more remarkable instances of the tenacity with which the active substance is adsorbed on to protein is shown in retention of activity of Soxhlet residues described below. This intense adsorption may partly account for the remarkable localization of sensitization when a tentacle is touched with dried skin or other 'insoluble' food.

Table 4

	Relative effect on neuromuscular system of tentacle	Relative sensitization of the cnidoblast to contact
Human saliva	+ + +	+ + +
Pecten mucus	+ + +	+ + +
Mucin (pure)	+	O
Soluble casein	+ + +	+ +
Fat-free casein	+ +	O
Egg white	+ +	+ +
Egg albumen (pure)	+ +	O
Gelatine	+	+
Flour	+	O
Amino acids (Table 2)	+ + to O	O
Sugars (Table 2)	O	O
Beef fat	O	O or ⊕
Egg yolk	+	O
Lecithin	+	+ +

Since the active substance is neither carbohydrate nor protein, though strongly adsorbed on the latter, it is natural to suggest that it falls in the general class of lipoids. To test this, a series of extracts was made from the gills and mantle of *Pecten maximus*. This tissue is very active in sensitizing the cnidoblasts to contact. Quantities of this substance were dried in vacuo and subjected to Soxhlet extraction with ether for several hours. The ethereal solution on evaporation to dryness gave a mass of fats. This fat produced no effect on cnidae whether it was allowed directly to touch the tentacles or whether the tentacles were immersed in a suspension of it. The 'fat-free' residue, on the other hand, remained as powerfully active in discharging the cnidae as the original food substance. This residue was then subjected to repeated washing with excess of distilled water. The washing fluid produced no obvious effect upon the cnidoblasts while pieces of the washed residue were still fairly effective in evoking a response. It thus appears that the active substance cannot be extracted by ether and confirms the fact that it is adsorbed strongly by the proteins.

The fat-free residue was then dried and subjected to Soxhlet extraction with ethyl alcohol for 3 or 4 hr. The dried residue from this extraction still produced some response of the cnidae when it touched the tentacles. On the other hand, evaporation of the

alcoholic extract yielded a hygroscopic waxy mass which produced a very powerful discharge when it made contact with a tentacle. This substance, when in suspension in sea water (0·1 %) sensitized the cnidoblasts to mechanical stimulation by a glass bead, just as in the case of food solutions. As might be expected, this substance did not show the reactions of protein to the ordinary tests (Biuret and Millon).

Soxhlet extraction with acetone of the dried residue from ether extraction also gives a highly active material. Thus, in one experiment a 103 g. wet weight of Pecten gill and mantle dried in vacuo weighed 10·5 g. (this includes about 3 g. of salts). Soxhlet extraction with ether gave 0·4 g. of fat which was ineffective on the cnidae. Soxhlet extraction with acetone of the fat-free residue gave 0·09 g. of active material. Soxhlet extraction with alcohol of the residue from this in turn gave 0·68 g. of highly active material. The final dry residue from this had lost a great deal of its effectiveness in discharging cnidae by contact, though it was still active to some extent. On the other hand, both the acetone and alcohol extracts were far more powerful than ordinary food and the slightest touch of the tentacle with a glass ball or even movement of the mucus near it produced a vast discharge (Fig. 3*b*). In spite of this, even strong solutions of this material only caused spontaneous discharge of an occasional cnida. In this there is complete resemblance to the action of true food substances and a marked distinction from the taurocholate group.

The nature of this active substance in the alcohol and acetone extracts has not yet been determined. Such an extract may be supposed to contain substances such as sterols and lecithin, etc. Cholesterol does not cause any obvious activation of the cnidae. A glass ball smoothly coated with it causes no marked discharge. Samples of lecithin, however (B.D.H.), prepared from hen's egg, are decidedly active in causing the discharge of the cnidae, though the activity of the samples tried was always less than that of alcoholic extracts of fresh food materials. Whatever the active substances may prove to be, it is significant that they are found in a fraction which includes highly surface-active substances. The other great group of cnidoblast stimulants, which include the bile salts, is also highly surface-active.

It thus appears that the substance which activates the cnidoblast to mechanical stimuli behaves as a lipoid soluble in alcohol and acetone and strongly adsorbed on to protein from whence it cannot be dislodged by ether.

THE SENSITIZATION OF THE CNIDOBLAST

The stimulus to the cnidoblast which causes discharge of the cnida is primarily mechanical contact. But normally this is only effective if certain chemical substances are present which lower the threshold of the cnidoblast to mechanical contact. These substances seem to be surface active lipoids, and it is their presence which accounts for the fact that the cnidae discharge into food though they do not generally do so into inert objects. We may in conclusion consider how the active substance sensitises the cnidoblast.

A food solution has the power of sensitizing the cnidoblasts to contact with an inert object such as a glass rod. It seems natural therefore to suppose that in the normal response the sensitizing substance diffuses through the sea water from the food to the cnidoblasts. This may actually take place when food surrounded by mucus and other juices reaches an anemone. Though since the sensitizing substance is strongly adsorbed on to protein and will not pass through a collodion membrane, it must be the colloidal particles on to which it is adsorbed that do the actual diffusing.

But the diffusion hypothesis will not cover all the facts. A piece of solid food, even if it is dried human skin, immediately causes a complete local discharge of cnidae from those cnidoblasts with which it comes in contact. No previous exposure of the anemone to food is necessary. This effect of solid food differs in several respects from that of food solutions. First, whereas sensitization by food solutions gradually increases over many minutes, sensitization by solid food is almost instantaneous and takes place simultaneously with contact. Second, unlike the general sensitization in a food solution, sensitization by solid food is entirely localized to those cnidoblasts with which contact is made. Touching neighbouring parts of the tentacle with a glass rod shows that there has been no diffusion of sensitization to neighbouring cnidoblasts, such as might be expected if there were diffusion of a dissolved sensitizer. Thirdly, solid food can be effective even though it is insoluble in water. Washed pieces of keratin or artificially denatured protein will induce a discharge. For a short time even glass beads freshly coated with paraffin wax will cause a small discharge. While therefore a food solution containing colloidal particles which bear the sensitizing agent can gradually sensitize the cnidoblasts over wide areas, quite insoluble substances can sensitize those cnidoblasts with which they come in direct contact, though they cannot affect those further away.

These facts indicate that the sensitizing agent can be conveyed to the cnidoblasts without passing into aqueous solution at all. There is a 'contact-chemical' stimulus. It is significant that the active agent appears to be a surface active lipoid and that its action is closely related to, though not identical with, that of a series of cytolytic agents like saponin which disorganize the cell membrane. It may be that sensitization is due to the direct conveyance to the cell membrane of surface-active lipoids in the food, and that their effect is to make this surface more easily excitable mechanically.

SUMMARY

1. The nature of the stimulus which causes discharge of the cnidae (nematocysts and spirocysts) in the tentacles of *Anemonia sulcata* has been studied.

2. The cnidoblasts behave as independent effectors. Contact with solid food or electric stimuli cause only local discharge. Repetitive electric stimulation at various frequencies causes spread of excitation far down the nerve net of the tentacle but the cnida discharge remains localized under the stimulating electrode. There is no physiological evidence for any connexion of cnidoblasts with the nervous system.

3. The stimulus to the cnidoblast is primarily due to direct mechanical contact. Discharge is easily effected by solid food. It can also be effected by inert solids provided the mechanical stimulus is sufficiently intense.

4. The normal stimulus to the cnidoblast is not a directly chemical one. Most food solutions and solutions of food derivatives do not cause a discharge. Some strong food solutions cause a few scattered cnidae to discharge. This differs from the intense local discharge to solid food.

5. Immersion in solutions of certain surface-active substances, such as the lower fatty acids, bile salts and saponin, causes an immediate general discharge of all the cnidae. This differs from the normal discharge to solid food.

6. Although natural food solutions do not normally cause a discharge, the cnidae are easily discharged by contact when they are present. That is, food solutions sensitize the cnidoblasts to mechanical stimuli.

7. The sensitizing substance is not protein. But it is strongly adsorbed on to protein. It cannot be extracted with ethyl ether. But it can be extracted with ethyl alcohol or acetone. Alcohol extracts of food are very active in sensitizing the cnidoblasts to mechanical disturbance.

8. Water insoluble foods can sensitize cnidoblasts with which they come in contact. Sensitization by contact is almost instantaneous. It is suggested that normally sensitization is due to some surface active lipoid directly transferred to the cnidoblast by contact.

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THE EFFECT OF CROWDING UPON THE OVIPOSITION OF GRAIN-INFESTING INSECTS

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(With Five Text-figures)

I

In a previous paper (Crombie, 1941) the factors which induce the beetle *Rhizopertha dominica* to oviposit were analysed. The experiments here outlined had for their purpose the investigation of the effect of competition and of population density upon the ovipositing behaviour of *Rhizopertha* and other insect pests of stored products. The influence of population density upon the fecundity of other insects, principally *Drosophila* and *Tribolium confusum*, has received extensive study at the hands of a number of biologists, summaries of whose results may be found in Pearl (1925), Allee (1934), Park (1937, 1939), Park & Woolcott (1937), Ford (1937) and Hammond (1938, 1939). From this work a number of general conclusions emerge, viz. that while slight crowding may stimulate, intense crowding usually depresses the rate of oviposition. This depression usually comes about through the operation, according to the circumstances, of any or all of the following agencies: 'jostling' or contacts between individuals, reduction in the amount of food available to individual animals, and 'conditioning' of the medium by the accumulation of various metabolic waste products. Furthermore, the operation of population density upon the rate of oviposition may occur at any stage in the life history of the individual concerned: thus overcrowded or underfed larvae may produce stunted and less fecund adults (Mackerras, 1933; Weidling, 1928), while in the adult instar crowding may influence feeding, rest, copulation or oviposition itself. The present investigation was planned under the influence of these ideas.

The following general precautions were taken in all the experiments below. All experiments were performed at 30° C. and a relative humidity of approximately 70%. These conditions were achieved in an incubator where conditions of darkness prevailed. The wheat used in all the experimental work was taken from a large stock of this material acquired at the beginning of the work. In order to standardize the water content it was then kept for not less than a month before use in an incubator at the experimental temperature and humidity, 30° C. and 70% R.H. (Bailey, 1920; Holdaway, 1932). The insects used for experimentation were in each case taken from well-fed, highly inbred stocks which were the descendants of a small number of original parents. The following insects, of which the biology has for the present purpose been adequately described in the literature, were used for experimentation: the beetles *Rhizopertha dominica* (Potter, 1935), *Oryzaephilus surinamensis* (Back & Cotton, 1926; Zacher, 1933; Thomas & Shepard, 1940), and *Acanthoscleides obtectus* (Zacher, 1933), and the moth *Sitotroga cerealella* (Back, 1922; Simmons & Ellington, 1924, 1925, 1933; Zacher, 1933).

In order to avoid confusion, the following definitions are given: *fecundity* is used to mean the rate of oviposition in terms of the total number of eggs laid per female per day.

Fertility means the number of fertile eggs oviposited per female per day. A fertile egg is one which hatches into a larva, and the term *egg-fertility* refers to the proportion of the eggs which do so.

II

We are concerned here, firstly, with the evaluation of the effect of population density, during both intra-specific and inter-specific competition, upon the fecundity and fertility of species which were used in experiments on the growth of pure and mixed populations; and secondly, with an attempt to analyse some of the physiological and behaviouristic factors involved. The second part of this programme was carried out chiefly with *Rhizopertha*, and the experiments upon this insect will be described first.

The effect of density upon oviposition rate in Rhizopertha. A number of experimental environments in dishes of standard size (6 cm. diameter and 1 cm. deep) were set up so that the density of *Rhizopertha* beetles (sex ratio unity) per wheat grain was in geometric series, as shown in Table 1. The beetles were less than 4 weeks old. Following the practice of Stanley (1941), the oviposition rates of the females were tested for a week immediately preceding the experiment, and the females whose fecundities lay outside the limits of 9–11 eggs per female per day were rejected. By this procedure difficulties arising from the great individual variation in the fecundity of insect females (cf. Chapman & Baird, 1934) were minimized, as were also inaccuracies due to the use of small samples. The wheat in each dish was accurately weighed, its water content having been standardized as described above, and in each of the grains two oblique cuts were made in a standard manner with a scalpel. This enabled the adults to feed, since they cannot attack intact wheat grains, and also provided oviposition sites for the females. The grains in all the experiments with *Rhizopertha* and *Oryzaephilus* were treated in this way. With up to 64 grains per dish the wheat was never more than one grain deep. For the 256 and 512 grain environments deeper dishes had to be used, but as shown later (vide infra) the size of the dish has no effect upon the rate of egg-laying of *Rhizopertha*. There were two dishes at each density, the whole experiment thus being duplicated.

The dishes were examined for eggs once every day for 8 days at approximately the same hour, and the following observations made: the number of eggs oviposited, the number of grains upon which eggs were oviposited, and the number of eggs oviposited upon the grains themselves. From the total number of grains used for oviposition we can calculate the average proportion of the grains used each day, and the average number of grains used per female per day. The eggs laid on the grains were usually found in the scalpel cuts mentioned above. After these observations had been made the contents of the dishes were emptied out. The beetles, with weighed fresh grains, were then replaced in the dishes and returned to the incubator. The material just emptied from the dishes was now sieved through No. 52 (extra quadruple) silk bolting cloth. This was fine enough to retain the eggs but allowed all the frass material to pass. The eggs were now removed from the material retained by the sieve, and discarded. The material retained by the sieve now consisted of the wheat alone. This was returned to the incubator so that its water content would remain constant. After 8 days all the used wheat was accurately weighed to the nearest 5 mg. on a chemical balance, and the rate of feeding under different conditions of density thus determined. The frass material passing through the sieve was also returned to the incubator each day and after 8 days weighed, thus providing a check on the previous figure.

We have thus controlled the physical environment, the food supply, and the conditioning of the medium. The number of insects per grain apparently remains the only variable. We have records of oviposition rate, rate of feeding, and the distribution of eggs in the environment. The fertility of the eggs was not determined in this experiment owing to the difficulty of collecting them from the grains without breaking. Fertility was measured in a later experiment however (Table 3). The results of the present experiment are set out in Table 1 and Fig. 1. The results of a *t*-test applied to this data are given under Table 1. The value of p (probability) represents the chance of obtaining as great a deviation by random sampling as that observed in the experiment. A probability of 5% is usually taken as the arbitrary division between significance and non-significance, so that when $p<0.05$ the difference between the values compared may be

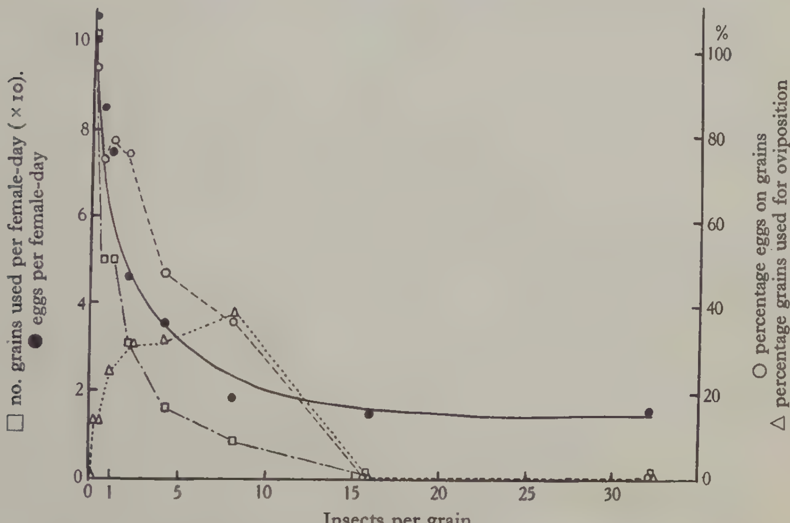

Fig. 1. The effect of crowding upon the fecundity of *Rhizopertha* (Table 1).

regarded as significant (Simpson & Roe, 1939). The results of all statistical tests in this paper are given in terms of the value of p, which possesses the same meaning in each case. The convention adopted here is that in setting out underneath each table the observations between which significant differences occur, the greater is where possible given first. (*a*) With densities above 0·25 beetle per grain (4 grains per beetle) fecundity falls as density increases up to 8 beetle per grain. (*b*) Within the range tested fecundity is not affected by changes in density below 0·25 beetles per grain. (*c*) As the density increases the proportion of wheat grains used per day for oviposition rises to a maximum at 1 beetle per grain, and falls to zero when density increases above 8 beetles per grain. (*d*) The number of grains used per female per day for oviposition falls with increase in density above 0·25 beetle per grain. The rate of egg laying was found to be very significantly correlated with the average number of wheat grains used for oviposition per female per day, the value of z corresponding to $p<0.001$. (*e*) The proportion of the eggs oviposited upon the grains themselves falls gradually with increasing density. Above 8 beetles per grain no eggs are oviposited upon the grains. (*f*) The rate of feeding

Table 1. *Effect of density upon the fecundity of* Rhizopertha

No. beetles	8	8	8	8	8	8	8	8	16	16	16	32
No. wheat grains	512	256	128	64	32	16	8	4	4	2	1	1
Beetles per grain	0·0156	0·0313	0·0625	0·125	0·25	0·5	1	2	4	8	16	32
Eggs counted on day 1	27	85	53	37	27	18	19	21	30	19	16	8
2	61	54	45	21	41	25	25	20	20	9	4	34
3	57	22	29	27	52	22	26	10	34	9	11	20
4	33	27	77	64	45	62	28	14	20	26	13	25
5	45	29	22	57	39	23	32	36	19	10	5	20
6	39	38	41	32	37	41	47	11	22	13	15	31
7	30	45	29	30	45	37	31	14	35	16	10	37
8	25	45	31	42	34	44	32	23	47	10	22	30
Total eggs	317	345	327	310	320	272	240	149	217	112	96	205
Eggs per female-day	9·9	10·8	10·2	9·7	10	8·5	7·5	4·7	3·4	1·8	1·5	1·6
Total no. grains used for oviposition	33	30	29	38	32	16	16	10	10	6	0	0
Average % grains used	0·8	1·5	2·9	7·4	12·5	12·5	25	31·25	31·25	37·5	0	0
Average no. grains used per female-day	1·03	0·94	0·91	1·19	1·0	0·5	0·5	0·31	0·16	0·09	0	0
% eggs on grains	100	92	97·6	90	94·9	73·8	76·2	74·3	47	36	0	0
No. mg. food eaten per 8 beetles per 8 days	130	110	110	90	120	90	130	110	110	49	23	13

Statistical analysis of Table 1

Significant differences were revealed by a *t*-test between

Fecundity at 0·25 and 1 beetle per grain	$0.02 < p < 0.05$
Fecundity at 1 and 2 beetles per grain	$0.02 < p < 0.05$
Fecundity at 4 and 8 beetles per grain	$0.02 < p < 0.05$
No. grains used at 0·125 and 0·5 beetle per grain	$0.02 < p < 0.05$
No. grains used at 1 and 2 beetles per grain	$0.02 < p < 0.05$
No. grains used at 2 and 4 beetles per grain	$p < 0.01$
No. grains used at 8 and 16 beetles per grain	$p < 0.01$
% grains used at 1 and 0·5 beetle per grain	$0.02 < p < 0.05$
% grains used at all adjacent densities below 0·25 beetle per grain: in each case	$0.02 < p < 0.05$
% eggs on grains at 0·25 and 0·5 beetle per grain	$p < 0.01$
% eggs on grains at 2 and 4 beetles per grain	$p < 0.01$
% eggs on grains at 8 and 16 beetles per grain	$p < 0.01$
Rates of feeding at 4 and 8 beetles per grain	$p < 0.01$

is not affected by crowding until a density of 8 beetles per grain is reached. At this and higher densities the rate of feeding is reduced.

Of the reasons why fecundity should fall with increasing density we may rule out conditioning of the medium, since this was controlled. It is unlikely that the accumulation of carbon dioxide in the crowded cultures was the cause of the reduced fecundity, for three reasons: firstly, the amounts of wheat were small; secondly, the number of beetles per dish was the same for all densities below 4 beetles per grain, yet fecundity had fallen before this density was reached; and thirdly, it is shown in a later experiment (vide infra) that reduction in the size of the dish, other factors being constant, has no effect upon fecundity. We may also rule out egg-eating as a cause of the observed results, since it does not occur in *Rhizopertha* when food is present.

We have seen that except at very high densities (above 4 beetles per grain) overcrowding does not reduce the rate of feeding. That is to say, starvation was not one of the causes of the reduction in fecundity from 10 to 3·4 eggs per female-day. At the density of 8 beetles per grain, however, when the rate of feeding is reduced by about half, fecundity falls to a value of 1·8. We cannot be sure, therefore, that this was not due to starvation. At 16 and 32 beetles per grain the rate of feeding is further reduced to approximately 20 and 10%, respectively, of the average maximum value, but fecundity undergoes no further significant fall from the value of 1·8. Therefore, over this range of density, reduction in the rate of feeding has no further effect upon fecundity. However, more experiments are clearly needed here. They will be described later (Table 2). It should be noted that, except for a slight deficiency at 32 beetles per grain, the amount of food present was at all densities sufficient to support the maximum rate of feeding. The average weight of a wheat grain was 50 mg., while the average maximum rate of feeding was 1·7 mg. per beetle per day. Therefore at the maximum rate of feeding 16 beetles would consume 27·2 mg. per day, and 32 beetles 54·4 mg. per day. The reduction in rate of feeding must have been due, therefore, not to any deficiency in the food supply, but to a reduction in the actual rate of eating due to interruption by other insects (cf. Pearl, 1932; Smirnov & Polejaeff, 1934).

Putting aside for the moment the possible effect of starvation at high densities, the following of the causes which suggest themselves as responsible for the reduction in fecundity with increasing density remain: competition for 'total space' with interruption of rest and of copulation, and competition for oviposition site resulting in the interruption of oviposition. From the experiment just described we have data concerning only the latter. Now we have observed, in Table 1, that with increasing densities above 0·25 beetle per grain, there is not only a gradual fall in fecundity, but also in the average number of grains used per female per day for oviposition, and in the proportion of eggs oviposited upon the grains themselves. All these effects begin at the same density, 0·5 beetle per grain. Now when fecundity is at the maximum rate the average number of grains used per female day for oviposition is one. But observation of the actual process of oviposition has shown that the females lay their eggs in groups, varying in size from about five to twenty eggs, all the eggs in one group being laid rapidly in succession, and that these periods of intense ovipositing activity are followed by periods during which no eggs are laid, or, if they are, only rarely and singly. Now since the females do not distinguish, for oviposition, between fresh grains and grains upon which eggs have already been oviposited (unpublished data), the eggs will be distributed at

random in space among the grains. The probability, at low densities, of the same grain being oviposited upon twice, is therefore extremely low. It seems likely, therefore, that all the eggs found upon one grain will have been laid in a group during a single period of intense oviposition, and, since the average number of grains used for oviposition per female per day is one, that these periods of intense oviposition recur after an average interval of 1 day. This was confirmed by an experiment in which ten females were confined separately in dishes containing ten wheat grains each. The grains were examined daily, those on which eggs were found being replaced by fresh grains. The experiment was continued for 6 days, at the end of which it was found that the average fecundity was 9 eggs per female-day, 84% of the eggs were oviposited on the grains, and the average number of grains used per female day was 0·96. In the sixty observations made, one grain was used per day for oviposition on forty-six occasions, two on six occasions, and on eight occasions no eggs were laid. Now the average number of grains used for oviposition per female-day, the fecundity and the percentage of eggs laid upon the grains themselves, remain fairly constant between densities of 0·0156 beetle per grain (64 grains per beetle) and 0·25 beetle per grain. Here we are observing the normal oviposition rhythm. With densities above 0·25 beetle per grain, however, the average number of grains used for oviposition per female-day falls. This is accompanied by a fall in the percentage of eggs oviposited upon the grains, and in fecundity. Thus it is clear that as density increases from 0·25 to 32 beetles per grain, the females behave as if competition for the wheat grains is becoming increasingly severe. They are forced to oviposit less frequently upon the grains, their oviposition rhythm is upset, and at high densities even feeding is affected. Competition for oviposition site (i.e. wheat grains) is therefore an important factor contributing to the reduction in fecundity by crowding.

The effect of copulation frequency upon fecundity and fertility. The interruption of rest and of copulation would, it appears, result from competition for 'total space'. Now the effect of density upon copulation frequency could influence fecundity, or fertility, only if copulation frequency itself influenced these variables. The following experiments were therefore carried out to investigate the effect of copulation frequency upon fecundity and fertility. First, the fecundity of virgin females was measured. Fifteen freshly emerged females were placed separately in dishes containing 2 g. flour and 8 false grains of plaster of Paris, each with a piece of paper attached to it (Crombie, 1941). The flour was sieved and renewed, and the false grains examined, every day until oviposition began. The average pre-oviposition period was thus determined. The flour was then sieved, and the eggs counted, every 2 days for 35 days from the first day of oviposition, and the fecundity of the virgin females thus measured. The eggs could be recovered without danger of breaking from this medium. They were incubated and the proportion of eggs hatching determined. Only fourteen out of the fifteen females oviposited. The average pre-oviposition period was 16·3 days, the shortest being 8 and the longest 26 days. The average fecundity was 0·67, lowest being 0·03 and highest 3·64 eggs per female-day. None of the eggs hatched. On the 35th day after the first egg was oviposited in each case one male was put with each female. The fecundity of each of the fourteen females increased greatly after an average interval of 2 days from copulation. The average fecundity for 7 days after the first day of increase after copulation was 9·96. Of the eggs laid after copulation 92·9% hatched. The female which had not oviposited as a virgin failed to oviposit after copulation also. The pre-oviposition period of females

which had been associated with males from emergence was determined in the same way. The average pre-oviposition period here was 5·6 days. The average fecundity and fertility for 7 days after the first oviposition were now determined. The average fecundity was 10·8, and 94·2% of these eggs hatched. It is clear that copulation shortens the pre-oviposition period and raises fecundity and fertility to a high value. The effect of frequency of copulation was now determined (cf. Park, 1933).

One hundred virgin females, 1 week old, were placed separately in dishes containing 2 g. flour and eight false grains. One male was placed with each for a period of 24 hr. The flour was sieved and renewed and the eggs counted every day. By the second day most of the females had commenced ovipositing rapidly, and the eggs were now counted every 2 days for a period of 8 days from the second day after copulation. That is to say, fecundity was recorded from the second to the tenth day after copulation. After this the females were now classified into groups according to fecundity over this 8-day period. Those falling into two groups, A with fecundity between 9 and 11 eggs per female-day, and B with fecundity between 5 and 6 eggs per female-day, were kept, the remainder of the females being discarded. By this procedure difficulties arising out of the great individual variation in fecundity were avoided. These particular fecundity limits were selected simply because a sufficient number of females fell within them. Each group was now subjected to identical treatment. Each was divided into two subgroups, (1) and (2). Subgroups (1) were kept as controls, eggs being counted every 2 days as before, and the percentage of the eggs hatching determined. Subgroups (2) were subjected to further copulation with males, as follows: during a second period of 8 days each female was associated with two males for 48 hr.; then, during a third period of 8 days, continuously associated with two males. Eggs were counted every 2 days, incubated, and the percentage hatching determined as before. Thus we have determined the fecundity and fertility of each group of females over three periods of 8 days each; in the first period the females had each been associated with one male for 24 hr., during the second with two males for 48 hr.; and during the third they were in continuous association with two males. It is to be noticed that any possible reduction in fecundity by population density has been avoided by using environments of sufficient size. The results were as follows: neither the average fecundity nor the egg-fertility of group A (2) during any of the three experimental periods differed significantly from those of group A (1), viz. 10·1 and 93·2%, respectively; nor did the average fecundity and egg-fertility of group B (2) during any of the three periods differ significantly from those of group B (1), viz. 5·8 and 96%, respectively. We may arrive therefore at the following conclusions: (1) the fecundity and fertility of copulated females is considerably higher than that of virgin females; (2) the pre-oviposition period of copulated females is shorter than that of virgin females; (3) neither fecundity nor fertility are affected by further copulations after the initial copulation. It is clear, therefore, that any effect density of population may have upon copulation frequency will have no effect upon fecundity and fertility.

In order to investigate the effect of 'total space' upon fecundity, the rate of egg-laying was measured as usual in dishes of three sizes, each containing thirty-two wheat grains and eight insects (sex-ratio unity) of the usual standard fecundity. The sizes of the dishes were as follows: (*a*) 20 cm. diameter and 5 cm. deep, (*b*) 6 cm. diameter and 1 cm. deep, and (*c*) 1·5 cm. diameter and 1 cm. deep. Fecundity in none of the dishes was significantly different from $10·9 \pm 0·78$ eggs per female-day, which was the value it assumed

in the largest dish. Nor did the average number of grains used for oviposition per day, nor the proportion of eggs deposited upon grains, differ significantly from 1·01 and 95%, respectively. The depressing effect of overcrowding upon fecundity therefore seems to depend upon the number of insects per wheat grain, and not per unit area or volume.

The effect of density upon copulation frequency. Although not immediately relevant to the present discussion, it was of interest, in view of MacLagan & Dunn's (1935) results with *Sitophilus oryzae*, to make a brief study of the effect of crowding upon copulation frequency. This was done as follows. In each of a series of dishes were placed eight wheat grains and a number of males and females, the males being marked with a spot of white paint on the elytra. Each dish was continuously observed for ½ hr. in a constant temperature room, and records kept of attempts to copulate and of male fights. The males often exchanged a few quick bites with the mandibles, after which they usually parted; each exchange of this sort was counted as 'a fight'. Two series of experiments

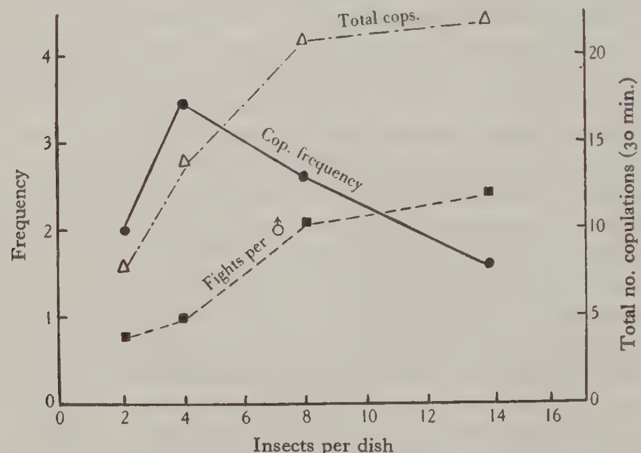

Fig. 2. The effect of crowding upon copulation frequency in *Rhizopertha*.

were performed. In the first the sex ratio remained at unity but the number of animals per dish was increased. The results are shown in Fig. 2. It will be noticed that there is an optimum density for female copulation frequency (copulations per female per ½ hr.), as MacLagan & Dunn found in *Sitophilus*, and that the amount of male fighting (fights per male per ½ hr.) increases with increasing density. Male copulation frequency of course coincided with that of the females and has not been shown separately. It appears that the increase in male fighting coincides with the decrease in copulation frequency. Now the males are the active partners in the act of mating; that is, they recognize the females and solicit them to mate, while the latter seem to remain indifferent (Crombie, 1941). The results seem to suggest, therefore, that male fighting is at least partly a result of direct competition for the females. This view is supported by the next experiment. Here, while the number of insects per dish remained constant at 12, the sex ratio was varied. The procedure was as before. The results are shown in Fig. 3. We observe that as the proportion of males to females increases, female copulation frequency rises to an optimum (at 2 males per female) and then falls, while male copulation frequency falls

continuously, and male fighting rises continuously. The absolute number of copulations per $\frac{1}{2}$ hr. falls almost continuously. Here the males are in much the same relation to the females, as, in the first experiment (Table 1), the females were to the wheat grains in which they were ovipositing. As the density of males per female increases, the frequency of male copulation decreases because of the competition of other males. Male fighting is a measure of this competition and we observe that it increases with increasing numbers of males. Furthermore, during the course of the experiments males were often observed to attack other males which were in the act of copulation, as well as to fight when they met in places where there were no females.

The effect upon fecundity of reduction of the amount of food while oviposition space remains constant. The following experiment was performed to determine whether the reduction of feeding rate at high densities would cause a reduction in fecundity there.

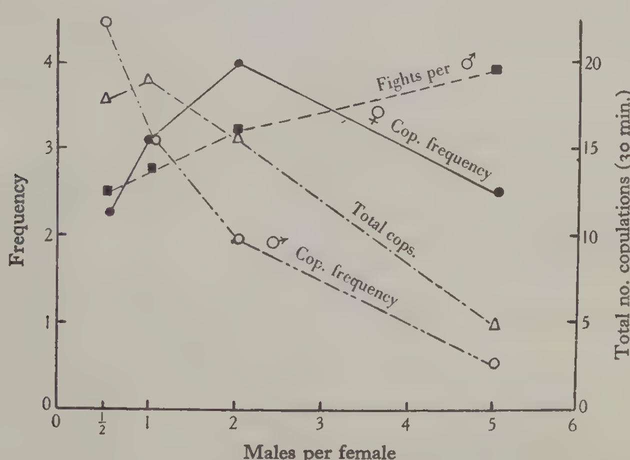

Fig. 3. The effect of the sex ratio upon copulation frequency in *Rhizopertha*.

Into each of a series of dishes a number of wheat grains and of false grains were placed. The latter were made of plaster of Paris, the same size and shape as the natural object, and each had a piece of paper attached to it to provide an oviposition site (Crombie, 1941). The total number of grains per dish remained constant at 32 (except at the highest density), while the proportion of wheat grains to false grains was altered as shown in Table 2. We have thus kept the oviposition space constant while reducing the food. Insects (sex-ratio unity) were now introduced into each dish in numbers such that the density of insects per wheat grain was in geometric series, while the density per grain, counting both wheat and false grains, remained constant at 0·25. The females, as before, were of standard fecundity 9–11 eggs per female-day. The procedure of the first experiment (Table 1) was now followed. The results are shown in Table 2. (*a*) Fecundity is not significantly reduced with densities up to 8 beetles per wheat grain, in spite of a fall in the rate of feeding at the latter density. (*b*) At 16 beetles per wheat grain fecundity falls to approximately 50%, while the rate of feeding also falls to approximately 25%, of the maximum values. (*c*) When the numbers of wheat grains and of false grains are approximately equal, preference is shown for the wheat grains

for oviposition, but with the increase in the proportion of false grains oviposition upon the latter occurs more frequently and upon wheat less frequently, until when there is only one wheat grain oviposition upon it almost ceases. As the density of insects per wheat grain increases, competition for the wheat becomes more severe and the females are forced to oviposit upon the false grains. False grains, however, appear to be perfect substitute sites (Crombie, 1941) and fecundity is not reduced until density reaches

Table 2. *Effect upon the fecundity of* Rhizopertha *of reducing food while oviposition space remains constant*

No. beetles	8	8	8	8	8	16
No. wheat grains	16	8	4	2	1	1
No. false grains	16	24	28	30	31	63
Beetles per wheat grain	0·5	1	2	4	8	16
Total eggs	280	304	314	276	286	344
Eggs per female-day	8·75	9·5	9·8	8·6	8·95	5·4
Total no. grains used for oviposition:						
(*a*) Wheat	36	20	20	15	4·5	1
(*b*) False	10	11	13	16	23	47
Total	46	31	33	31	27·5	48
Average no. grains used per female-day:						
(*a*) Wheat	1·13	0·63	0·62	0·47	0·14	0·02
(*b*) False	0·31	0·34	0·41	0·50	0·72	0·74
Total	1·44	0·97	1·03	0·97	0·86	0·76
Average % grains used	18	12	13	12	10·8	18·8
% eggs on grains:						
(*a*) Wheat	75·7	72·2	48·1	34·6	16·8	1·1
(*b*) False	19·3	27·1	43·7	47·4	51·5	70·0
Total	95·0	99·3	91·8	82·0	68·2	71·1
No. mg. wheat eaten per 8 beetles per 8 days	97	95	130	64	51	31

Statistical analysis of Table 2

Significant differences were revealed by a *t*-test between

Fecundity at 8 and 16 beetles per wheat grain	$p<0{\cdot}01$
% eggs on wheat grains at 0·5 and 4 beetles per wheat grain	$p<0{\cdot}01$
% eggs on wheat grains at 8 and 16 beetles per wheat grain	$p=0{\cdot}05$
% eggs on false grains at 4 and 0·5 beetles per wheat grain	$p=0{\cdot}05$
% eggs on wheat and on false grains at 0·5, 8 and 16 beetles per wheat grain, respectively: in each case	$p<0{\cdot}01$
Rates of feeding at 8 and all densities below 2 beetles per wheat grain	$0{\cdot}02<p<0{\cdot}05$

The following were compared by means of χ^2:

No. wheat grains used at different densities and the average over the whole range	$p<0{\cdot}01$
No. false grains used at different densities and the average over the whole range	$p=0{\cdot}05$
No. wheat grains used and the no. false grains used	$p<0{\cdot}01$
Fecundity in Tables 2 and 1 over the same range of density	$p<0{\cdot}01$
No. grains used in Tables 2 and 1 over the same range of density	$p<0{\cdot}01$

16 insects per wheat grain, nor is the normal oviposition rhythm upset. Approximately one grain is used for oviposition per female-day, and the high proportion of eggs laid upon grains undergoes no significant fall. (*d*) The reduction in fecundity at 16 beetles per wheat grain must have been due to starvation, since all other factors were controlled. At 8 insects per wheat grain the rate of feeding was reduced, but apparently not sufficiently to have an effect upon fecundity. It should be noted, however, that the rate of feeding, measured as here by the amount of frass material removed from the grains, is probably not a measure of the rate at which the insects actually ingest the food, since much of the frass material is simply pushed behind the animals as they burrow into

the grains. But dissections of females which had been at densities of 8 and 16 showed that they were suffering from starvation (vide infra).

Apart from the question of rest, all the suggested causes of the reduction of fecundity with increasing density have now been eliminated except for interruption of oviposition through competition for the grains and, at very high densities only, starvation. Some experiments were now performed to carry this analysis still further.

The effect upon fecundity and fertility of reducing oviposition space while food remains constant. In this experiment, 'oviposition space' and 'feeding space', normally localized together upon the wheat grains, were separated. This was done by taking advantage of the fact that the false grains of plaster appear to be just as acceptable as the genuine article for purposes of oviposition. A series of experimental environments were set up in dishes each of which contained 0·4 g. flour, while the number of false grains varied

Table 3. *Effect upon the fecundity and fertility of* Rhizopertha *of reducing oviposition space while food remains constant at 0·4 g.*

No. beetles	8	8	8	8	8	8	8
No. false grains	32	16	8	4	2	1	0
No. beetles per grain	0·25	0·5	1	2	4	8	—
Total eggs	352	260	237	211	129	144	125
Eggs per female-day	11	8·1	7·4	6·6	4·0	4·5	3·9
Total no. false grains used for oviposition	20	20	17	11	5	2	—
Average % grains used	7·8	15·4	26·6	34·4	31·3	25	—
Average no. grains used per female-day	0·63	0·63	0·53	0·34	0·15	0·06	—
% eggs on grains	51	45	49·7	31·4	14·8	11·8	—
% eggs hatching	97·3	91·8	99·2	95·7	94·9	96·4	98·2

Statistical analysis of Table 3

Significant differences were revealed by a t-test between	
Fecundity at 0·25 and 1 beetle per grain	$p<0{\cdot}01$
Fecundity at 2 and 4 beetles per grain	$0{\cdot}01<p<0{\cdot}02$
No. grains used at 1 and 4 beetles per grain	$p<0{\cdot}01$
% grains used at 0·5 and 1 beetle per grain	$p=0{\cdot}05$
% eggs on grains at 1 and 4 beetles per grain	$p=0{\cdot}05$
The following were compared by means of χ^2:	
No. grains used at different densities and the average over the whole range	$p<0{\cdot}01$
Fecundity in Tables 3 and 1 from 2 to 8 beetles per grain	$0{\cdot}02<p<0{\cdot}05$
No. eggs on grains in Tables 1 and 3	$p<0{\cdot}01$
No. grains used in Tables 1 and 3	$0{\cdot}02<p<0{\cdot}05$

from dish to dish. Eight insects (sex-ratio unity) were introduced into each dish so that the densities of insects per false grain were in geometric series as shown in Table 3. As before, the females were of standard fecundity 9–11 eggs per female-day. There were two dishes at each density. Thus the food supply remained constant (0·4 g. flour) while the 'oviposition space' was varied, and furthermore, feeding and oviposition took place in different parts of the environment. The flour was sieved and the grains examined every day for 8 days, and the total number of eggs, the number of eggs oviposited upon the grains, and the number of grains used for oviposition, counted. The eggs were then incubated and the percentage hatching determined. The sieve used was No. 52 (extra quadruple) silk bolting cloth which retained the eggs while allowing the flour to pass through (cf. Chapman, 1918). The eggs could be recovered without danger of breaking by this means.

The results are given in Table 3. (*a*) Fecundity falls with increasing density of beetles per false grain, even though the amount of food remains constant. (*b*) Approximately

coinciding with the fall in fecundity there is a fall in both the average number of false grains used for oviposition and in the percentage of eggs oviposited upon these grains. (*c*) The fertility of the eggs remains unaffected by the crowding of the females. (*d*) Fecundity here is not significantly different from fecundity at the corresponding densities in wheat (Table 1) until 1 beetle per grain is reached. With densities greater than this value fecundity here is significantly higher than in Table 1. Now fecundity here does not fall below the value (4·0) reached at 4 beetles per grain, even in the complete absence of grains, and even at 0·25 beetle per grain, when fecundity is maximum, only 51% of the eggs are oviposited on the grains, and only 0·63 grain is used per female-day for oviposition. The flour thus forms an important oviposition site. Two more observations, however, should now be mentioned. The first is that the addition of extra flour, even up to 1 g. per beetle, to dishes containing no false grains did not cause the fecundity to rise significantly above 4 eggs per female-day. And conversely, the reduction of the amount of flour present in dishes containing two false grains per beetle to as little as 2 mg. per beetle per day (which is sufficient to prevent starvation, cf. Table 1) did not cause a fall in fecundity below the average maximum, 10 eggs per female-day. The second was made as follows: Ten females, of standard fecundity 9–11, were confined separately in dishes containing 0·05 g. of flour each. Two false grains were placed in each dish, and eggs counted and flour removed daily for 4 days. This procedure was then continued for a second period of 4 days without false grains in the dishes, a third period with false grains, and a fourth period without false grains. The average fecundities during each of these four periods were 8·8, 3·6, 9·4 and 5·4 eggs per female-day, respectively. The average fecundity with and without false grains were compared by calculating the χ^2 values, and found to be very significantly different ($p < 0{\cdot}01$). Therefore, without any possibility of competition, the absence of false grains results in a considerable fall in fecundity. This is undoubtedly because without false grains the tactile sensations necessary to stimulate oviposition are absent (Crombie, 1941). This factor must also be operating in the experiment shown in Table 3, but the fall in fecundity while there are still false grains present, combined with a fall in the number of grains used and in the proportion of eggs laid on the grains, indicates that here competition for the false grains is also effective in reducing the rate of oviposition. We may therefore conclude that competition for oviposition site, without any interference by feeding animals, causes a reduction in fecundity.

Differential effect of males and females on fecundity. Three experiments were performed. In the first, the number of males present with one female in a series of dishes containing 8 wheat grains was varied so that the densities of beetles per grain were in geometric series (Table 4). The females were of standard fecundity 9–11 eggs per female-day as before, and there were eight dishes (i.e. eight females) at each density. Otherwise the procedure was as in the first experiment (Table 1). The results are given in Table 4. Fecundity falls with increasing numbers of males, but fecundity here is significantly *higher* than that over the same range of density with sex-ratio unity (Table 1). However, the competition of the males does reduce female fecundity, significant differences in fecundity occurring between 0·25 and 4 beetles per grain, and in the proportion of eggs laid upon grains between 0·5 and 2 beetles per grain, respectively. In the second experiment the procedure was the same but there were no males present. Density was increased by the addition of females only, as shown in Table 5. As before, we observe that

fecundity falls with increasing density, but in this case the fecundity is significantly *lower* than that over the same range of density with sex ratio unity (Table 1). Significant differences occur in Table 5 between fecundity at 0·25 and 0·5, and at 1 and 2 beetles per grain; and between the proportions of eggs laid upon grains at 0·25 and 0·5, and at 0·5 and 2, beetles per grain, respectively. Now the reduction in fecundity in Table 4

Table 4. *Effect of male density upon the fecundity of* Rhizopertha *females (eight dishes at each density; 8 days)*

No. males	1	3	7	15	15
No. females	1	1	1	1	1
No. wheat grains	8	8	8	8	4
Beetles per grain	0·25	0·5	1	2	4
Average total no. eggs per dish	76·8	72·8	70	62	49
Eggs per female-day	9·6	9·1	8·7	7·8	6·1
% eggs on grains	95	89·2	77·1	60	64

Statistical analysis of Table 4

Significant differences were revealed by a t-test between
- Fecundity at 0·25 and 4 beetles per grain — $p < 0.01$
- % eggs on grains at 0·5 and 2 beetles per grain — $0.02 < p < 0.05$

The following were compared by means of χ^2:
- Fecundity in Tables 4 and 1 over the same range of density — $p < 0.01$

may have been due either to the competition of the males for the grains during feeding, or to the interference of the *Rhizopertha* males with the females of the same species by trying to copulate with them. A third experiment was therefore performed in which the arrangement of Table 4 was repeated but *Oryzaephilus* males, which also feed upon the grains, were substituted for *Rhizopertha* males. The results are shown in Table 6,

Table 5. *Effect of female density upon the fecundity of* Rhizopertha *(two dishes at each density except the lowest where there were four; 8 days)*

No. females	2	4	8	16
No. wheat grains	8	8	8	8
Females per grain	0·25	0·5	1	2
Average total no. eggs per dish	150	212	326	384
Eggs per female-day	9·4	6·6	5·1	3
% eggs on grains	97·2	65	52·5	39·1

Statistical analysis of Table 5

Significant differences were revealed by a t-test between
- Fecundity at 0·25 and 0·5 beetle per grain — $0.02 < p < 0.05$
- Fecundity at 1 and 2 beetles per grain — $0.02 < p < 0.05$
- % eggs on grains at 0·25 and 0·5 beetle per grain — $0.02 < p < 0.05$
- % eggs on grains at 0·5 and 2 beetles per grain — $0.02 < p < 0.05$

The following were compared by means of χ^2:
- Fecundity in Tables 1 and 5 over the same range of density — $p < 0.01$

significant differences occurring between fecundity at 1 and 4, and between the proportion of eggs laid upon grains at 0·5 and 4 beetles per grain, respectively. When fecundities in Tables 4 and 6 were compared by calculating the value of χ^2, $p > 0.2$, so that there is no significant difference between them. Therefore, although *Oryzaephilus* males interfere only by feeding, and *Rhizopertha* males by both feeding and copulating, the quantitative effect on the fecundity of the females is the same.

We have omitted so far any discussion of the effect of interruption of 'rest' upon fecundity. This was one of the factors postulated by Pearl (1932) as causing the reduction in fecundity of *Drosophila*. It seems unlikely that this would be important in the case of *Rhizopertha*. It is possible that the insects may be fatigued by becoming excited and made to move about more by the presence of other insects. But there seems to be no reason why males should excite the females less than the latter excite each other; in fact one would expect it to be the other way round; yet the presence of females had a considerably greater influence upon fecundity than that of males. Any effect crowding may have in the way of increasing fatigue would therefore seem to be relatively unimportant in relation to fecundity.

The conclusions to which we may now arrive are as follows: (1) Males contribute to the effect of density upon the rate of oviposition of the females because they compete, for the purpose of feeding, for the grains in which the females oviposit. (2) But the most important factor (at any rate up to the density of 4 insects per grain) responsible for the reduction in the rate of oviposition of the females here is competition for the grains by the ovipositing females themselves. Competition for parts of the environment

Table 6. *Effect of* Oryzaephilus *males upon the fecundity of* Rhizopertha *in wheat* (*eight dishes at each density;* 8 *days*)

No. *Oryzaephilus* males	3	7	15	15
No. *Rhizopertha*	1	1	1	1
No. grains	8	8	8	4
Beetles per grain	0·5	1	2	4
Average total no. eggs per dish	81·6	70·3	57·6	46·4
Eggs per female-day	10·2	8·8	7·2	5·8
% eggs on grains	97	79·6	79·5	68·8

Statistical analysis of Table 6

Significant differences were revealed by a *t*-test between
Fecundity at 1 and 4 beetles per grain $p=0·02$
% eggs on grains at 0·5 and 4 beetles per grain $p=0·05$

other than the grains is apparently without effect upon oviposition rate. Females compete for the grains for both food and oviposition site. It seems possible that while at the lower densities it is the ovipositing activity of the gravid females which causes most interference with egg laying, at higher densities the struggle for the grains for feeding purposes is the more important. It is to be noted that on the average never more than approximately one-third of the grains present is actually used for oviposition (MacLagan & Dunn, 1935). (3) At 16 insects per grain crowding is so intense that the rate of feeding is reduced below starvation level (Table 2) with adverse effects upon reproduction. (4) With the conditions described, no other factors appear to have any effect upon the rate of oviposition of *Rhizopertha*. (5) In all these experiments both the fall in the rate of oviposition with intense crowding, and the rise following the reduction of crowding, were found to take place immediately upon subjection to these conditions. In every case fecundity returned to the maximum immediately upon the return to a sufficiently low density. The effect of crowding seems therefore to have been temporary and behaviouristic, rather than permanent and more deeply physiological. (6) Egg-fertility is not affected by adult density. (7) Neither fecundity nor fertility is influenced by copulation frequency.

III

Effect on fecundity of competition between two insects for different niches in the same environment. The bruchid beetle *Acanthoscleides obtectus*, when given the choice, oviposits upon beans to the exclusion of wheat grains (unpublished data). *Rhizopertha*, on the other hand, under the same circumstances almost always chooses the wheat (Crombie, 1941). In an environment containing wheat and undamaged beans *Rhizopertha* and *Acanthoscleides* would compete, therefore, only for 'total space'. As a control the effect of density upon fecundity in homogeneous populations *Acanthoscleides* alone was first determined. Experimental environments were set up in a series of dishes in such a way that the number of *Acanthoscleides* (sex-ratio unity) per bean was in geometric series as shown in Table 7. The beetles, which are easily sexed (Zacher, 1933), were freshly emerged. The beans were undamaged with intact tests, and there were two dishes at each density. The dishes were examined for eggs after 8 days in the incubator and the usual counts made. The results are given in Table 7. Significant differences occur between fecundity at 1 and 2 beetles per bean, and between the percentage of eggs laid upon the beans at 0·5 and 4 beetles per bean. Most of the eggs, however, were at all densities oviposited loosely among the beans and were not attached to them. There was no regular change in egg-fertility with increased crowding of the adults.

Table 7. *Effect of density upon the fecundity and fertility of* Acanthoscleides

No. beetles	2	4	8	16	32
No. beans	4	4	4	4	4
Beetles per bean	0·5	1	2	4	8
Total eggs	35	58	74	115	115
Eggs per female-day	4·4	3·6	2·3	1·8	0·9
% eggs on beans	28	22·2	19	7·7	13
% eggs hatching	65	74	93	76	58

Statistical analysis of Table 7

Significant differences were revealed by a t-test between
Fecundity at 1 and 2 beetles per bean $0{\cdot}01 < p < 0{\cdot}02$
% eggs on beans 0·5 and 4 beetles per bean $p = 0{\cdot}05$

Rhizopertha with its food, wheat, and *Acanthoscleides* with beans, were then brought together into the same environment. Each dish contained 8 wheat grains and 4 beans together with equal numbers of *Rhizopertha* and *Acanthoscleides*, so that the densities of *Rhizopertha* per wheat grain and of *Acanthoscleides* per bean were, respectively, in geometric series as shown in Table 8. The sex ratio in both species was unity. The *Acanthoscleides* were freshly emerged, while the *Rhizopertha* females were of standard fecundity 9–11 eggs per female-day as before. The dishes were examined after 8 days in the incubator, and observations made of the numbers and positions of eggs oviposited, as before. The results are given in Table 8. Except at very high density the effect of crowding upon the fecundity of each species is quantitatively the same as in the previous experiments (Tables 1 and 7) when it alone was present. There are no significant differences between the fecundity of *Rhizopertha* at corresponding densities in Tables 1 and 8 except at 4 beetles per grain, at which fecundity in the latter table is significantly lower. Similarly, the fecundity of *Acanthoscleides* follows the same course in Tables 7 and 8 except at 8 beetles per bean, where fecundity is significantly lower in the latter table. Each species, it appears, oviposits on its own natural food to the exclusion of

the other food present, and except with very intense crowding there is no interference between them. The change in oviposition rhythm of *Rhizopertha* with increasing density, as shown by the number of grains used for oviposition per female-day, is similar to that seen in Table 1, when this species was by itself. The values of this variable in the two tables over the same range of density were compared by calculating the value of χ^2, which corresponded to $p > 0{\cdot}1$, showing that there is no significant difference between them. However, although there is no competition for oviposition site, the presence of the *Acanthoscleides* does affect the distribution of eggs by *Rhizopertha*. Here increased crowding produces no fall in the proportion of eggs oviposited upon the grains, such as occurs in Table 1. The explanation of this may be that *Acanthoscleides*, by competing

Table 8. *Effect of competition for different ecological niches upon the fecundity of* Rhizopertha *and* Acanthoscleides

	Rhiz.	*Acan.*	*Rhiz.*	*Acan.*	*Rhiz.*	*Acan.*	*Rhiz.*	*Acan.*	*Rhiz.*	*Acan.*
No. insects	2	2	4	4	8	8	16	16	32	32
No. wheat grains	8	—	8	—	8	—	8	—	8	—
No. beans	—	4	—	4	—	4	—	4	—	4
No. beetles per specific natural seed	0·25	0·5	0·5	1	1	2	2	4	4	8
Total eggs	81	38	144	66	239	86	256	147	83	47
Eggs per female-day	10·1	4·8	9	4·1	7·5	2·7	4	2·3*	0·8	0·4
No. grains used per female-day	1·1	—	0·7	—	0·75	—	0·3	—	0·12	—
% eggs on wheat	87	1	75	5·6	81·3	0·3	84·3	0	86·4	0
% eggs on beans	0	24	0	17	3·1	20·1	0	12·5	0	8·2

Statistical analysis of Table 8

Significant differences were revealed by a t-test between

Fecundity of *Rhizopertha* at 1 and 2 beetles per grain	$p < 0{\cdot}01$
Fecundity of *Rhizopertha* at 2 and 4 beetles per grain	$p = 0{\cdot}02$
Fecundity of *Acanthoscleides* at 1 and 4 beetles per bean	$p = 0{\cdot}05$
Fecundity of *Acanthoscleides* at 4 and 8 beetles per bean	$0{\cdot}01 < p < 0{\cdot}02$
Fecundity of *Rhizopertha* in Tables 1 and 8 at 4 beetles per grain	$0{\cdot}02 < p < 0{\cdot}05$
Fecundity of *Acanthoscleides* in Tables 7 and 8 at 8 beetles per bean	$p < 0{\cdot}01$
% *Rhizopertha* eggs on grains in Tables 8 and 1 at 4 beetles per grain	$p < 0{\cdot}01$

for 'total space', forces the *Rhizopertha* females to keep to the wheat for oviposition whatever the density. This experiment confirms our previous conclusion, that competition for oviposition site is the most important factor in reducing fecundity.

Effect of density upon fecundity of Oryzaephilus *in wheat*. The arrangement of this experiment was identical with that for *Rhizopertha* shown in Table 1. In a series of dishes the numbers of beetles (sex-ratio unity) and of wheat grains were varied so that density was in geometric series as shown in Table 9. The wheat grains each had two scalpel cuts made in them as before, and the females were of standard fecundity 3–4 eggs per female-day. The beetles were less than a month old. The dishes were examined, the usual observations being made, and the grains renewed, once a day for 8 days. The results are given in Table 9. The fall in fecundity which occurs at densities above 0·5 insect per grain is accompanied by a fall in the number of grains used for oviposition per female-day, and in the percentage of eggs oviposited upon the grains themselves. The normal oviposition rhythm of *Oryzaephilus* females was observed independently by confining seven of them separately in dishes each containing 10 wheat grains. The dishes were examined daily, grains on which eggs were found being replaced by fresh

ones. The oviposition rhythm was in some ways different from that of *Rhizopertha*. Days on which two or more grains were used alternated with days on which no eggs were laid by individual females. At the end of 6 days the average fecundity was 2·6 eggs per female-day, of which 96% were found in the cuts in the grains. The average number of grains used per female-day was 1·46. In the 42 observations made, 1 grain was used per day on 11 occasions, 2 grains 11 times, 3 grains 6 times, 4 grains 4 times, and on 10 occasions no eggs were laid. The number of grains used shows a highly significant positive correlation with the number of eggs laid per day. The calculated value of z corresponds to $p < 0{\cdot}001$. It seems then that, as with *Rhizopertha*, the reduction in fecundity with increased crowding shown in Table 9 is accompanied by an alteration in the distribution of eggs in the environment and in the oviposition rhythm of the females. These observations can only be explained by assuming that there was intense competition for the wheat for purposes of oviposition. With this insect, however, the

Table 9. *Effect of density upon the fecundity of* Oryzaephilus *in wheat*

No. beetles	8	8	8	8	8	8	8
No. wheat grains	64	32	16	8	4	2	1
Beetles per grain	0·125	0·25	0·5	1	2	4	8
Total eggs	106	102	96	54	42	32	35
Eggs per female-day	3·3	3·2	3·0	1·7	1·3	1	1·1
Total no. grains used for oviposition	58	67	58	28	17	4	2
Average no. grains used per female-day	1·8	1·5	1·8	0·88	0·53	0·13	0·06
% eggs on grains	96·7	97·2	91·3	72·6	52·5	37	12·9

Statistical analysis of Table 9

Significant differences were revealed by a t-test between

Fecundity at 0·5 and 1 beetle per grain	$p < 0{\cdot}01$
No. grains used at 0·5 and 1 beetle per grain	$0{\cdot}02 < p < 0{\cdot}05$
No. grains used at 2 and 4 beetles per grain	$0{\cdot}02 < p < 0{\cdot}05$
% eggs on grains at 0·5 and 2 beetles per grain	$0{\cdot}02 < p < 0{\cdot}05$
% eggs on grains at 1 and 4 beetles per grain	$0{\cdot}02 < p < 0{\cdot}05$
% eggs on grains at 2 and 8 beetles per grain	$0{\cdot}02 < p < 0{\cdot}05$

practice of egg-eating by the adults may account for part, at least, of the reduction of eggs with increasing density. It is possible that the eggs oviposited in the cuts in the grains were protected from this cannibalism, and that while the number of eggs oviposited each day remained constant, greater numbers were oviposited in the open and consequently eaten as the number of wheat grains decreased. We observe, however, that at the highest density where, if this were true, all the eggs in the open would be eaten, only 12·9% of the eggs were found upon the grains, so that egg-eating cannot have had a very important effect.

Effect upon the fecundity of both species of competition between Rhizopertha *and* Oryzaephilus *in wheat*. The above experiment was now repeated except that at each density half the insects were *Rhizopertha* and half *Oryzaephilus* as shown in Table 10. The sex-ratio unity in each species was unity, and the females were of the standard fecundities 9–11 and 3–4 eggs per female-day, respectively. The results are given in Table 10. The fecundity of both species falls as usual with increasing density, but in neither does the fecundity at any density differ significantly from that when that species is alone (Tables 1 and 9). The distribution of eggs in the environment is also affected by density, but for each species there is no significant difference between the numbers of grains used for oviposition, or between the proportions of eggs oviposited upon the grains, when that

species is alone and when it is in competition with the other. While the fall in fecundity with increased crowding is thus accompanied by the usual evidence of intense competition for oviposition site, the effect upon each species of the competition of members of the other species appears to be quantitatively identical with that of the competition of the same number of its own species.

The effect of density upon fecundity and fertility in Sitotroga cerealella. This insect is short lived, copulates within a few hours of emergence, and lays almost all its eggs within the first 3 days of its adult life (Simmons & Ellington, 1933). Females will oviposit as many eggs through gauze or between two pieces of cardboard held together with a paper clip as among wheat grains (Simmons & Ellington, 1924), and the adults do not feed. Experimental environments were set up as follows: small crystallizing dishes, of 6 cm. diameter and 5 cm. depth, were used. The open rim was ground level

Table 10. *Effect of competition in wheat for the same ecological niche upon the fecundity of* Rhizopertha *and* Oryzaephilus

	Rhiz.	*Oryz.*	*Rhiz.*	*Oryz.*	*Rhiz.*	*Oryz.*	*Rhiz.*	*Oryz.*
No. insects	4	4	4	4	8	8	16	16
No. wheat grains	16		8		8		8	
Insects per grain	0·5		1		2		4	
Total eggs	166	50	102	34	134	42	230	51
Eggs per female-day	10·4	3·1	6·4	2·1	4·5	1·3	3·6	0·8
Total no. grains used for oviposition	18	20	9	14	5	5	4	3
Average no. grains used per female-day	1·11	1·25	0·55	0·88	0·31	0·31	0·25	0·19
% eggs on grains	85	83·9	94·5	65·9	82·4	54·2	70·7	32·4

Statistical analysis of Table 10

Significant differences were revealed by a t-test between

Fecundity of *Rhizopertha* at 0·5 and 1 beetle per grain	$p<0·01$
Fecundity of *Rhizopertha* at 1 and 2 beetles per grain	$p<0·01$
% *Rhizopertha* eggs on grains at 1 and 4 beetles per grain	$p=0·05$
Fecundity of *Oryzaephilus* at 0·5 and 1 beetle per grain	$0·02<p<0·05$
Fecundity of *Oryzaephilus* at 1 and 2 beetles per grain	$0·01<p<0·02$
% *Oryzaephilus* eggs on grains at 0·5 and 1 beetle per grain	$0·01<p<0·02$
% *Oryzaephilus* eggs on grains at 1 and 4 beetles per grain	$p<0·01$

The following were compared by means of χ^2:

No. grains used by *Rhizopertha* at different densities and the average over the whole range	$p<0·01$
No. grains used by *Oryzaephilus* at different densities and the average over the whole range	$p<0·01$

and the dishes were stood upside down upon ground glass plates, the rim fitting flush with the plate. This was necessary to prevent the females inserting their eggs between the rim of the dish and the plate (cf. Ellington, 1930). In a series of such dishes experimental environments were set up such that the number of moths (sex-ratio unity) per wheat grain (intact) was in geometric series as shown in Table 11. There were two dishes at each density when there were eight or more moths per dish. Otherwise there was a number of dishes such that at each density eight females were tested. The insects were freshly emerged. The dishes were examined for eggs once a day until all the moths were dead, the grains being renewed daily. The following records were kept: the number of eggs per dish, the number of eggs oviposited upon or under the wheat grains, the number of grains upon which eggs had been oviposited, and the number and sex of moths found dead each day. The latter were removed. The eggs were incubated and

Table 11. *Effect of density upon the fecundity and fertility of* Sitotroga (*eight dishes at first three densities; four at next four; two at last six*)

No. insects	2	2	2	4	4	4	4	8	8	16	16	32	64
No. grains	128	64	32	32	16	8	4	4	2	2	1	1	1
No. insects per grain	0·0156	0·0313	0·0625	0·125	0·25	0·5	1	2	4	8	16	32	64
Eggs counted on day 1	98	72	60	119	134	129	70	134	110	160	180	270	380
2	27	49	45	74	83	79	95	161	130	256	235	240	600
3	11	12	14	37	41	37	30	80	34	140	95	280	450
4	6	4	4	5	9	7	11	8	7	51	23	80	150
5	1	4	4	3	7	6	6	4	3	25	7	20	50
Average total eggs per dish (5 days)	143	137	120	251	274	258	213	388	302	632	540	890	1630
Eggs per female (5 days)	143	137	120	125	137	129	106	97	75	79	68	56	51
Average total no. grains used per dish for oviposition (5 days)	10·4	11	9·4	21·3	15	21·8	20	20	10	10	5	5	5
Average no. grains used per female in 5 days	10·4	11	9·4	10·7	7·5	10·9	10	5	2·5	1·25	0·625	0·313	0·156
% eggs on grains	100	100	100	100	100	98·2	94·7	79·2	57	39·1	21·4	23·6	23
% eggs hatching	97·1	94·3	91·7	98·1	95·5	96·2	94·8	95·5	97·3	92·8	96·4	97·9	90·9

Statistical analysis of Table 11

Significant differences were revealed by a *t*-test between

Rates of oviposition at 0·5 and 2 moths per grain	$p = 0·05$
Rates of oviposition at 2 and 4 moths per grain	$0·02 < p < 0·05$
Rates of oviposition at 1 and 16 moths per grain	$p < 0·01$
% eggs on grains at 1 and 4 moths per grain	$0·01 < p < 0·02$
No. grains used at all adjacent densities above 1 moth per grain: in each case	$p < 0·01$

The following were compared by calculating d/σ:

Daily % eggs laid at 0·0156 and 1 moth per grain	$p < 0·01$
Daily % eggs laid at 1 and 64 moths per grain	$p = 0·05$

the percentage hatching determined. Oviposition had practically ceased by the 5th day so that only the eggs laid during the first 5 days of life were included in calculating the rate of oviposition, this variable being expressed in terms of eggs per female over the whole 5 day period. The results are given in Table 11. The rate of oviposition decreases with increasing densities above 1 insect per grain, and coinciding with this there is a decrease in the average number of grains used per female for oviposition, and in the percentage of eggs oviposited upon the grains themselves. The insects prefer to oviposit upon or under the grains, at low density each female using about 2 grains per day for oviposition. This value was approximated over the whole 5 day period; the size of the egg-batches grew smaller as the females grew older, but on each day the average number of grains used per female was approximately 2. On the last days grains were often found on which only one egg had been oviposited. From this it may possibly be inferred that *Sitotroga* females have an intense ovipositing period twice in 24 hr. At the density of 1 moth per grain there were of course 2 grains per female and we observe that each day all of the grains were used for oviposition. With increasing densities above 1 moth per grain there were less than 2 grains per female, and competition for the grains became increasingly severe. The few grains present were covered with eggs. Rather than retain their eggs the females now oviposited away from the grains (usually these eggs were attached to the glass where the top of the dish met the ground-glass floor or placed under dead individuals), but this resulted in a fall in the rate of oviposition. Further evidence of the pressure for oviposition space at high densities is afforded by the distribution of the eggs over the 5 days of oviposition. While at the lowest density 60·3% were oviposited on the first day, 27·7% on the second, 6·2% on the third, 3·4% on the fourth and 2·4% on the fifth day, at densities above 0·5 insect per grain the proportion of the eggs oviposited during the later days was much higher. Thus at the highest density 23·3% of the eggs were oviposited on the first day, 36·8% on the second, 27·6% on the third, 9·2% on the fourth and 3% on the fifth. Intermediate densities had intermediate distributions. We notice that the rate of oviposition decreases with increasing numbers of insects per grain, and not merely with increasing numbers per dish. This suggests that competition for 'total space' is unimportant, and since the grains are used for no other purpose than oviposition it seems probable that the rate of oviposition is reduced through competition for the grains only during the act of oviposition. The fertility of the eggs was not affected by adult density.

As in *Drosophila* (Pearl & Parker, 1922; Pearl, Miner & Parker, 1927) longevity was found to decrease with increasing density, a significant difference occurring, however, only between the densities 4 and 64 insects per grain. The value of t here corresponds to 0·02 ($p < 0{\cdot}05$). There was no significant difference between the longevity of males and females, which is not in agreement with the results of Simmons & Ellington (1924), who found that under optimum conditions females lived slightly longer than males.

Effect of competition between Rhizopertha *and* Sitotroga *upon the fecundity of both species.* Experimental environments were set up in five dishes of the same type as those just described. Each dish contained 4 wheat grains (each with two cuts as above), and 4 *Rhizopertha* and 4 *Sitotroga* (thus making 2 insects per grain), the sex ratio in each species being unity. The *Sitotroga* were freshly emerged and the *Rhizopertha* females of standard fecundity 9–11 eggs per female-day as before. The dishes were examined at approximately the same hour each day for 6 days and the usual observations taken.

The grains were renewed each day. The results are given in Table 12. The rate of oviposition of *Sitotroga* over the first 5 days was 129·5 eggs per female, which is not significantly different ($p > 0{\cdot}1$) from the average maximum rate (128·9) reached at optimum densities in the absence of *Rhizopertha* (Table 11). *Rhizopertha* at this density does not therefore reduce the egg-laying rate of *Sitotroga*. But over the first 2 days, during which the *Sitotroga* oviposited 73 % of their eggs, the fecundity of *Rhizopertha* was 5·15 eggs per female-day, which is significantly lower than that during the last 4 days (8·1 eggs per female-day), during which period the *Sitotroga* oviposited only 27 % of its eggs. The fecundity of *Rhizopertha* over the last 4 days (8·1) is, furthermore, not significantly different ($p > 0{\cdot}1$) from that (7·5 eggs per female-day) at the same density of *Rhizopertha* per grain in the absence of *Sitotroga* (Table 1). It is clear therefore that during its period of most rapid oviposition *Sitotroga* reduces the fecundity of *Rhizopertha*, while from the third day onwards, when the *Sitotroga* females oviposit much less, the *Rhizopertha* females are not seriously interfered with. This is confirmed by the observation that on the third day there occurs a statistically significant rise in both the average number of grains used per female per day for oviposition, and in the proportion of eggs oviposited upon the grains. The eggs of the two species were often found side by side, indicating that there was no avoidance of the other's eggs by the gravid females of each species. This conclusion that the fecundity of the *Rhizopertha* was reduced by competition for the grains by the ovipositing *Sitotroga* females is supported by the results of an experiment in which the *Rhizopertha* were competing under the same conditions with *Sitotroga* males only. Here the average fecundity of the *Rhizopertha* was 7·8 eggs per female-day, which does not differ significantly ($p > 0{\cdot}1$) from the 7·5 eggs per female-day oviposited with 1 *Rhizopertha* per grain in the absence of *Sitotroga* males (Table 1).

IV

A brief study was made of the effect of homotypic and heterotypic 'conditioning' of the medium upon fecundity and fertility. The technique was similar to that used by Park (1936, 1937) with *Tribolium*. 'Conditioned medium' was taken from cultures in which the population had just died out. It was sieved through No. 52 (extra quadruple) silk bolting cloth, and the part passing through the sieve used for experimentation. The number of animals which had inhabited these cultures was known from counts made at regular intervals while studying the course of population growth in unrenewed media.

Effect of homotypically conditioned media upon the fecundity and fertility of Rhizopertha. Experimental environments were set up such that while each dish contained 3·2 g. flour, the proportion of conditioned flour increased, while the proportion of fresh flour decreased, as shown in Table 13. To this were added two more dishes in which no conditioned flour was present. One contained 0·1 g. of fresh flour while the other contained no food at all, as shown in the last two columns of Table 13. Each dish contained 64 false grains so that there was no reduction in fecundity by overcrowding. There were duplicate experimental dishes for each degree of conditioning. Sixteen insects (sex-ratio unity) were introduced into each dish, the females being of standard fecundity 9–11 eggs per female-day as before. Following the practice of Park (1934) a parallel set of environments was set up, and insects which died in the experimental dishes were replaced by insects from the parallel dishes. The latter insects would have been under the same conditions as those in the experimental dishes. Except with complete starvation, however,

Table 12. *Effect of* Sitotroga *upon the fecundity of* Rhizopertha *in wheat* (*4 wheat grains per dish*)

Day	1		2		3		4		5		6		Total	
	Rhiz.	*Sit.*	*Rhiz.*	*Sit.*	*Rhiz.*	*Sit.*	*Rhiz.*	*Sit.*	*Rhiz.*	*Sit.*	*Rhiz.*	*Sit.*	*Rhiz.*	*Sit.*
Average no. insects	4	4	4	4	4	4	4	3·2*	4	2·8*	4	0·4*	—	—
No. insects per grain	1	1	1	1	1	1	1	0·9	1	0·7	1	0·1	—	—
Eggs found in dishes 1	10	90	12	90	8	35	14	15	19	5	19	4	82	239
2	12	140	14	90	21	65	18	19	20	5	11	0	96	319
3	8	108	10	60	18	35	11	15	15	7	20	4	82	229
4	11	118	12	70	12	40	12	15	18	7	14	0	79	250
5	10	114	4	61	18	60	20	21	19	10	18	0	89	266
Total eggs	51	570	52	371	77	235	75	85	91	34	82	8	428	1303
Eggs per female-day	5·1	57	5·2	37·1	7·7	23·5	7·5	10·6	9·1	4·3	8·2	4	7·1	130·3†
Average no. grains used per female-day for oviposition	0·47	—	0·54	—	0·89	—	0·97	—	1·4	—	1·01	—	—	—
% eggs on grains	57·2	97·9	69·1	98·7	91·2	94·7	93·7	100	98·9	100	99·1	100	—	—

* *Sitotroga* begin to die. † Eggs per female per 5 days.

Statistical analysis of Table 12

Significant differences were revealed by a *t*-test between

Fecundity of *Rhizopertha* during days 3–6 and 1–2	$p < 0{\cdot}01$
No. grains used per day by *Rhizopertha* on days 3 and 2	$p = 0{\cdot}02$
% *Rhizopertha* eggs on grains on days 3 and 2	$p = 0{\cdot}05$

mortality was not heavy in these experiments. The flour was sieved, the eggs removed and counted, and the same flour replaced, once every 4 days for 20 days. The eggs were incubated and the percentage hatching determined. On the twentieth day the insects from each dish were placed in 3·2 g. of fresh flour (with 64 false grains) and the same observations made until fecundity had returned to the control value. Thus was obtained data concerning the effect of differentially conditioned media upon fecundity, fertility and rate of recovery, and of starvation (without conditioned medium) upon the same variables. The results are given in Table 13 and Fig. 4. A significant fall in fecundity,

Table 13. *Effect of differentially, homotypically conditioned flour upon the fecundity and fertility of* Rhizopertha

No. insects	16	16	16	16	16	16	16	16	16	16
No. grams flour:										
(*a*) Fresh	3·2	2·8	2·4	1·6	0·8	0·4	0·2	0	0·1	0
(*b*) Conditioned	0	0·4	0·8	1·6	2·4	2·8	3·0	3·2	—	—
% conditioned flour	0	12·5	25	50	75	87·5	93·75	100	—	—
No. eggs counted on day 4	354	266	235	136	126	43	89	52	360	89
8	287	94	134	155	127	103	93	80	276	91
13	322	174	142	81	102	114	61	89	360	12
16	246	120	119	54	92	142	85	55	303	0
20	293	82	50	165	113	160	120	124	234	0
Total	1502	732	680	591	560	562	448	400	1533	192
Eggs per female-day	9·4	4·6	4·25	3·7	3·6	3·5	2·8	2·5	9·6	1·2
(fresh flour on day 20) 24	291	180	240	200	192	140	188	168	354	0
28	308	350	380	370	288	332	340	307	—	28
32	—	—	—	—	—	—	—	—	—	128
36	—	—	—	—	—	—	—	—	—	300
Eggs per female-day over last 4 days	9·7	10·9	11·9	11·6	9	10·4	10·6	9·6	11·1	9·4
% eggs hatching	91·7	98·2	95·4	97·6	98·2	95·7	96·1	92·9	90·6	89·1

Statistical analysis of Table 13

Significant differences were revealed by a *t*-test between

- Fecundity over days 0–20 in fresh flour and in 12·5, 25, 50 and 87·5 % conditioned media: in each case — $0.02 < p < 0.05$
- Fecundity over days 0–20 in fresh flour and all other conditioned media: in each case — $p < 0.01$
- Fecundity over days 0–4 in 25 and 50 % conditioned media — $p = 0.02$
- Fecundity over days 0–4 in 75 and 87·5 % conditioned media — $0.02 < p < 0.05$
- Fecundity over days 0–4 and 4–20 in 12·5 % conditioned media — $p < 0.01$
- Fecundity over days 0–4 and 4–20 in 25 % conditioned media — $0.01 < p < 0.02$
- Fecundity over days 20–24 in fresh flour and any of the conditioned media: in each case — $p < 0.01$
- Fecundity in fresh flour and with complete starvation over days 20–24, 24–28 and 28–32: in each case — $p < 0.01$

measured over the 20 day period, occurs between fresh medium and medium containing 12·5 % conditioned flour. There is no further fall in fecundity measured over this period, even though the proportion of conditioned medium increases up to 100 %. But if fecundity be measured over the first 4 days only, significant differences occur between media containing 25 and 50 %, and 75 and 87·5 % conditioned flour, respectively, while there is no significant difference between fecundity in fresh flour and in medium containing only 12·5 % conditioned medium. Thus at first the reduction in fecundity is roughly proportional to the degree of conditioning, but later fecundity in all conditioned

media falls to the same level (cf. Park, 1936; Park & Woolcott, 1937). With 12·5 and 25% conditioned medium fecundity over the first 4 days is significantly greater than that over the remaining 16 days. There is no significant difference in rate of recovery in any of the conditioned media, but the fecundity of insects from all the conditional media during the first 4 days after replacement in fresh flour is significantly lower than that in fresh flour. By the eighth day reading, however, all have completely recovered. Egg-fertility was not affected by conditioned medium.

Effect of heterotypically conditioned media upon the fecundity and fertility of Rhizopertha. The arrangement of this experiment was identical with that of the previous except that flour conditioned by *Oryzaephilus* and *Sitotroga*, respectively, were substituted for the *Rhizopertha*-conditioned flour, as shown in Table 14. The conditioned medium was taken, as before, from cultures in which the population had just died out, the total number of insects which had lived in it being known as before from population counts. The results are given in Table 14 and Fig. 4. Significant differences occur between the following: fecundity in fresh flour (Table 13) and in any of the conditioned media; in 50 and 100% *Oryzaephilus* media; in 50% *Oryzaephilus* medium and 100% *Sitotroga* medium; in 12·5% *Rhizopertha* medium (Table 13) and 100% *Sitotroga* medium; and in 100% *Rhizopertha* medium and 50 and 100% *Oryzaephilus* media, respectively. Thus except for the last mentioned, where the depressing effect of the *Oryzaephilus* medium was slightly greater, the heterotypically conditioned media have the same quantitative effect upon the fecundity of *Rhizopertha* as those homotypically conditioned to the same degree. Also, the heavier the conditioning the greater the depression of fecundity (Fig. 4). The fecundity of insects from both *Oryzaephilus* media remain significantly lower than that in the control (Table 13, fresh flour) up to 8 days after replacement in fresh flour. By the twelfth day recovery is complete. On the other hand recovery from the *Sitotroga* medium is complete in 4 days. Thus recovery from *Oryzaephilus* media is slower, and from *Sitotroga* media faster, than that from homotypically conditioned media. Egg-fertility was, as before, unaffected.

There came to mind two possible causes of the depression of fecundity by conditioned media, viz. starvation and 'poisoning' (Park, 1934). Now a population living in a limited amount of food will eventually die out: the question then arises whether it dies out because it is poisoned or because it is starved. Populations had died out in the conditioned media used here, so that heavier conditioning would not be encountered in nature. Several pieces of evidence support the view that the major cause of the depression of fecundity in *Rhizopertha* was poisoning. First, the fall in fecundity caused by only 12·5% conditioned medium is, in spite of the presence of ample fresh food, as great as that caused by more heavily conditioned media even up to 100%, although the time for the conditioned medium to take effect is longer with light than with heavier conditioning. The second piece of evidence comes from the data in the last two columns of Table 13. In the second last column there is only 0·1 g. of flour per dish. This flour was renewed daily to prevent conditioning. Although there is less fresh flour here than in the 93·75% conditioned medium, fecundity remains at its maximum and indeed we know from the data on rate of feeding in Table 1 that the insects would not be starving. It is possible of course that the fresh flour mixed with conditioned media is less easily available to the insects than it is here, but taken with the other evidence this observation suggests that it is the presence of conditioned medium, rather than the absence of food,

Table 14. *Effect of heterotypically conditioned flour upon the fecundity and fertility of* Rhizopertha

No. insects	16	16	16
No. grams flour	3·2	3·2	3·2
Conditioned flour from:	*Oryzaephilus*		*Sitotroga*
% conditioned flour	50 %	100 %	100 %
No. eggs counted on day 4	128	41	82
8	190	24	52
13	132	64	46
16	90	30	76
20	100	33	48
Total	640	192	304
Eggs per female-day (fresh flour on day 20)	4·0	1·2	1·9
24	214	173	332
28	234	243	—
32	288	285	—
Eggs per female-day over last 4 days	9	8·9	10·4
% eggs hatching	95·4	97·2	94·9

Statistical analysis of Table 14

Significant differences were revealed by a *t*-test between

Fecundity in fresh flour (Table 13) and in any of the conditioned media: in each case	$p<0.01$
Fecundity in 50 and 100 % *Oryzaephilus* media	$p<0.01$
Fecundity in 50 % *Oryzaephilus* and 100 % *Sitotroga* media	$0.01<p<0.02$
Fecundity in 12·5 % *Rhizopertha* (Table 13) and 100 % *Sitotroga* media	$p=0.05$
Fecundity in 50 % *Oryzaephilus* and 100 % *Rhizopertha* media (Table 13)	$0.02<p<0.05$
Fecundity in 100 % *Rhizopertha* and 100 % *Oryzaephilus* media	$0.01<p<0.02$
Fecundity over days 20–24 and 24–28 in fresh flour and in either of the *Oryzaephilus* media: in each case	$0.02<p<0.05$

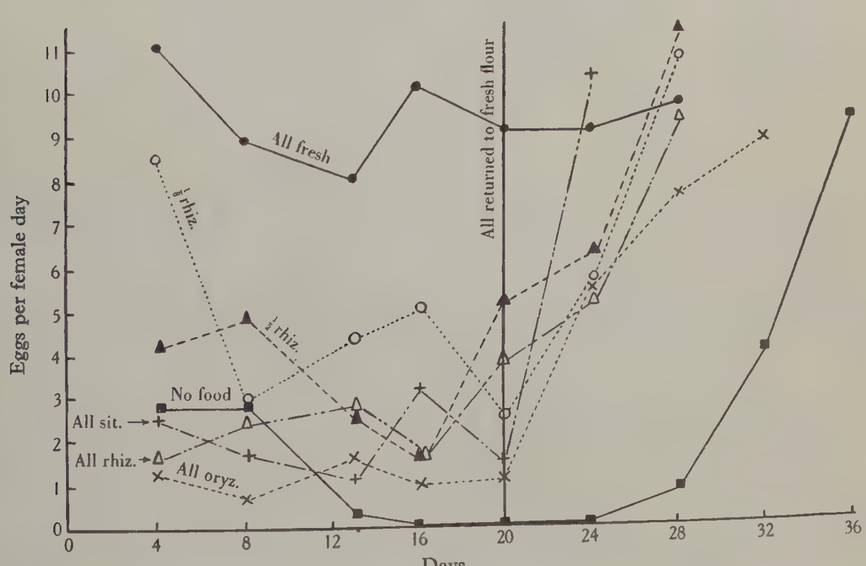

Fig. 4. The effect of conditioned media upon the fecundity of *Rhizopertha* (Tables 13 and 14).

which depresses fecundity. The effect of complete starvation upon fecundity (Table 13, last column) is different in several respects from that of the conditioned media. No eggs are laid after the thirteenth day, and the rate of recovery on being given fresh food is much slower than that from any of the conditioned media. Furthermore, mortality became very heavy after about 14 days, and nearly half the animals had to be replaced from the parallel dishes. Starvation, however, did not depress egg-fertility.

Now we have seen that a number of conditions will depress the rate of oviposition of *Rhizopertha*, viz. the lack of suitable oviposition sites, mutual interference owing to competition for oviposition sites with overcrowding, conditioning of the medium, and starvation. Females which had been subjected for 14 days to the various conditions described in the previous experiments were dissected and their gonads examined. According to Nüsslin & Rhumbler (1922) the presence of corpora lutea, together with eggs in the paired oviducts, indicates that egg-production is in progress, while corpora lutea alone, without eggs in the paired oviducts, indicate that egg-production has occurred

Table 15. *The results of dissecting* Rhizopertha *females exposed to various conditions*

Exp. conditions	No. females	Average fecundity	No. females containing eggs on dissection %	Eggs found on dissection			Corpora lutea
				Total	Average per female	Range	
(A) Fresh flour + false grains (control)	40	9·1	36 = 90	296	7·4 ± 0·9*	0–26	+
(B) Fresh flour only (restraining)	16	4·5	14 = 88	75	4·7 ± 2·0	0–14	+
(C) False grains only (starving)	23	1·4	7 = 30	26	1·13 ± 0·37	0– 6	+
(D) 16 beetles per grain—partially starving	15	2·7	12 = 80	87	5·8 ± 2·4	0–13	+
(E) 12·5 % conditioned medium	14	3·3	10 = 71·4	60	4·3 ± 1·7	0–11	+
(F) 100 % conditioned medium	15	2·7	10 = 66·7	79	5·3 ± 1·4	0–17	+

* Standard error.

but has now ceased (Dick, 1937). These statements were in most cases confirmed by our observations, although in a few insects in which continuous oviposition indicated that egg-production was in progress, no eggs were found in the paired oviducts, while on the other hand a few eggs were found in some of the starving females which were not ovipositing. The appearance of the ovule tubes was also usually different according to whether oviposition was or was not occurring. In the former case they were large and white, and in the latter small and transparent. The following groups of females were dissected, the results appearing in Table 15: (A) In the controls, from environments containing fresh flour and false grains (Tables 3 and 13), eggs were found in 36 out of 40 (90 %) females of which the average fecundity had been 9·1 eggs per female-day, and the average number of eggs found per female on dissection was 7·4 ± 0·9, the range being 0–26. Corpora lutea were present here and in all the other females dissected during this experiment (Fig. 5A). Here also, and elsewhere except in the starving females, the ovule tubes were large and white, and the fat in the body cavity abundant and translucent. (B) The average fecundity of females which had been exercising

restraint in environments containing fresh flour but no false grains was reduced to 4·5. The average number of eggs found on dissection per female (4·7 ± 2·0) is, however, not significantly different from that in the controls, since the difference between them is less than twice the standard error of either. Restraint therefore had no permanent effect upon egg-production. (C) Eggs were found in only 7 out of 23 (30%) females which

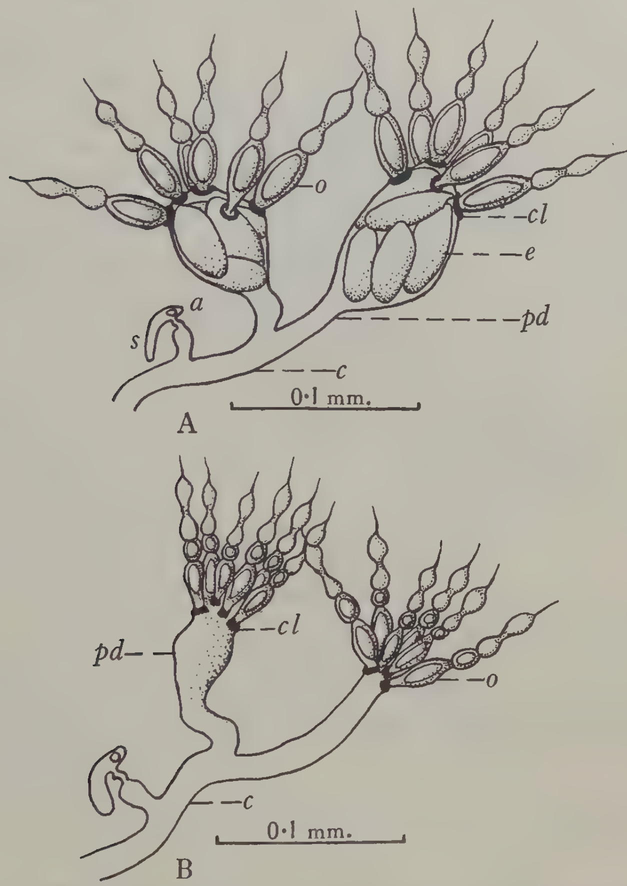

Fig. 5. Changes in the reproductive organs of females of *Rhizopertha dominica*. A. Normal condition of organs of females in which oviposition is in progress. B. Condition of organs in females in which oviposition has occurred, but has now ceased as a result of complete starvation. *a*, accessory gland; *c*, common oviduct; *cl*, corpus luteum; *e*, egg; *o*, ovariole; *pd*, paired oviduct; *s*, spermatheca.

had been starving for 14 days (Table 13, last column). Here the average fecundity was 1·4. The average number of eggs found per female on dissection was 1·13 ± 0·37, which is significantly lower than the value of this variable in the controls (A), viz. 7·4 ± 0·9. The scanty fat had a white curdled appearance which was characteristic of starving animals. The ovule tubes were usually small and transparent, indicating that egg-production had ceased (Fig. 5*B*). (D) With females whose rate of feeding had been reduced to approximately 20% of the normal rate by the high density to which they

were exposed (Tables 1 and 2, last columns), the average fecundity was reduced to 2·7, but the average number of eggs found on dissection per female ($5·8 \pm 2·4$) is not significantly lower than that in the controls. The fat was reduced in quantity and had a white, curdled appearance. Thus, although these females bore the marks of starvation, egg-production had not been affected. The average number of eggs found on the dissection of females from (E) 12·5 % conditioned medium ($4·3 \pm 1·7$ per female) is not significantly different from the number found either in the controls or in females from (F) 100 % conditioned medium ($5·3 \pm 1·4$ per female). Nor are the two last-mentioned values significantly different. Therefore, although conditioned medium reduces the rate of oviposition, neither conditioning nor the degree of conditioning appear to affect egg-production. The fat was normal in females from both conditioned media, indicating that little or no starvation has occurred here. This observation, together with the evidence already mentioned, suggests very strongly that it is some 'poisoning' effect, and not starvation, which is responsible for the depression of fecundity in conditioned media.

Now the signs indicate that in all except the completely starving females (C) egg-production is continuing and eggs are being oviposited, while in (C) egg-production has occurred, but has now ceased. Only in (C) is the number of eggs found per female significantly lower than that found in the controls (A). In all other cases the difference from (A) is less than twice the standard error of either. In (C) also the proportion of females containing eggs is much lower than in the other groups. We have seen that the lack of suitable oviposition sites, and the mutual interference of ovipositing females at high density, are behaviouristic in their effect upon fecundity, and that their effects last only so long as these conditions prevail. In these two cases then the females refrain from oviposition even though they have the eggs to lay. Starvation, on the other hand, has its effect primarily upon egg-production, so that the insects cannot lay eggs, even though they would (Fig. 5 B), and recovery is not immediate. In (D) the behaviouristic effects of overcrowding, and starvation, are both in operation, although the immediate recovery of maximum fecundity upon the reduction of density suggests that the former is the most important. Our imperfect knowledge of the physiological action of conditioned media makes it impossible to decide how the 'poisoning' there would operate, for although most of the evidence supports the view that the ovipositing females may exercise restraint on finding themselves in such unsuitable environments, rather than that egg-production is affected, the slow recovery after replacement in fresh flour indicates that this is different from the other cases in which the effect of the experimental conditions is primarily upon behaviour.

When cultures of *Rhizopertha*, *Oryzaephilus* and *Sitotroga* are grown in wheat with the same physical conditions as defined here, and the food is kept fresh and its mass constant by regular renewal, the maximum population densities reached by the three species are approximately 1·7, 2·1 and 0·9 adults per grain, respectively (unpublished). At these densities the fecundity of the first two species has the values 4·7–7·5 and 1·3 eggs per female-day, respectively, while *Sitotroga* is still ovipositing at its maximum rate of over 100 eggs per female in 5 days. The longevity of *Sitotroga* remains at these densities also unaffected. Thus in populations of *Rhizopertha* and *Oryzaephilus* the reduction in fecundity through overcrowding makes an important contribution to the effect of density upon survival. Similarly, the conditioning of the medium, such as would occur in environments where excretory products are allowed to accumulate and

the food is not renewed, makes an important contribution to the same effect in cultures where *Rhizopertha* is either living alone or competing with *Sitotroga* or *Oryzaephilus*. Important as these factors are, however, even at the extremes of adverse conditions possible in actual populations fecundity remains of such magnitude that a considerable mortality must occur in the immature stages before the offspring are reduced to numbers commensurate with the capacity of the environment to support them.

SUMMARY

The crowding of adults invariably had a depressing effect upon the rate of oviposition in the insects studied here, while egg-fertility was not affected. In unconditioned media, at densities possible in actual populations, the reduction of fecundity was, it appears, entirely a result of competition for the oviposition sites usually for two purposes, viz. oviposition and feeding. That is to say, at such densities the effect of crowding upon oviposition was of a behaviouristic nature. When two species were living in the same environment their mutual effect upon each other's fecundity was more or less dependent upon the degree of identity of the niches for which they were competing. The reduction of fecundity of *Rhizopertha* by homotypically and heterotypically conditioned media was at first roughly proportional to the degree of conditioning, but after a time the effect of all media was the same. Homo- and hetero-typical conditioning were not radically different in effect. It is believed that conditioned medium operates upon fecundity through 'poisoning', and that the effect is upon oviposition rather than, as that of starvation, upon egg-production. When returned to an optimum environment the insects recovered from all the experimental conditions mentioned here: immediately from conditions of overcrowding, more slowly from conditioned media, and more slowly still from complete starvation.

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